IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number	:	10/577,074	Confirmation No.: 9018
Applicant	:	James M. Cregg *et al.*	
Filed	:	April 24, 2006	
Title	:	METHODS OF SYNTHESIZING HETEROMULTIMERIC POLYPEPTIDES IN YEAST USING A HAPLOID MATING STRATEGY	
TC/Art Unit	:	1636	
Examiner:	:	Nancy T. VOGEL	
Docket No.	:	67858.701501	
Customer No.	:	**21967**	

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. LEON GARCIA-MARTINEZ UNDER 37 C.F.R. § 1.132

Sir,

I, Leon Garcia-Martinez, Ph.D., declare that:

1.) I am currently employed as the Director of Antibody Therapeutics at Alder Biopharmaceuticals, Inc., the assignee of all right, title and interest in the above-captioned patent application number 10/577,074 ("the '074 application").

2.) I was awarded my Ph.D. in 1993 from the University of Texas at Austin in the field of Biochemistry. I subsequently spent five years as a post-doctoral fellow at the University Of Texas Southwestern Medical Center studying the transcriptional regulation of the HIV-1 and HIV-2 virus. My educational history is provided in my *Curriculum Vitae* ("CV"), provided herewith as the CV Appendix.

3.) From 1998 until I joined Alder Biopharmaceuticals, Inc., in September 2005, I was employed at Signal Pharmaceuticals (now part of Celgene Corporation), at Celltech R&D (now part of UCB), and at ICOS Corporation. Since joining Alder Biopharmaceuticals, Inc., in September 2005, I have directed the company's activities in the area of fermentation

research. This has included the development of bio-processes for the expression of full length monoclonal antibodies using our diploid *Pichia pastoris* strains, as well as the transfer of the technology to a contract manufacturing organization for its scale-up. The expression of full-length antibodies using Alder Biopharmaceutical's diploid *Pichia pastoris* strains has been successfully scaled to 2000L.

4.) I have read, and am familiar with, the specification and claims of the '074 application as well as the Office Action issued on March 18, 2009 (hereinafter the "Office Action"), as well as all of the documents specifically identified in the Office Action and in this Declaration.

5.) I understand based on this review that claim 21 of the '074 application has been rejected in the Office Action "... as claiming subject matter which was not described in the application in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention." I have been advised that "enable" is a legal term and in the context of the rejection means that the Examiner has preliminarily concluded that the teachings of the specification considered with technical information that would have been generally known in the field of the subject invention (yeast fermentation) would allegedly not teach a skilled artisan how to practice the claimed methods absent significant ("undue") experimentation. More specifically, I note that the Examiner has expressed a viewpoint that it would not be routine to produce 100 mg/l of complete (intact) antibodies in recombinant yeast. I further understand that the Examiner has expressed a viewpoint that the amount of guidance provided in the '074 application is limited to the construction of the yeast cells, and it is the Examiner's position that there is no further guidance in the '074 application as to how to obtain the high level of production claimed.

6.) I have been asked (based on my relevant experience in the use of microbial fermentation to produce recombinant proteins, and in particular my familiarity regarding the use of *Pichia* to produce recombinant proteins such as antibodies) to provide my opinion as to whether one of ordinary skill in the art would have been able to produce a complete antibody at levels of at least 100 mg/l or higher as of October 22, 2003, by expressing same in a diploid yeast strain, e.g., a *Pichia* strain, following the teachings described in the '074 application and further based on information generally known to those familiar with yeast fermentation absent the exercise of "undue experimentation". For the reasons contained

herein, and further based on the experimental evidence provided herewith, my opinion is overwhelmingly "yes".

7.) I must note at the outset that my opinion does not in anyway contradict the truly unexpected nature of this result (that diploidal *Pichia* using conventional fermentation techniques would have been capable of expressing intact antibodies at such high levels). This was truly unexpected since yeast such as *Pichia* normally exists in haploid form. A more plausible result and conclusion without the benefit of hindsight concerning the present invention would have been that diploidal *Pichia* would have been less favored for the production of intact antibodies and that the yields of intact antibody produced thereby would be negligible and/or that the diploidal yeast cells might not be capable of secreting intact antibody into the culture medium. It would not have been anticipated prior to the subject invention that diploid yeast such as *Pichia* would have been capable of stably expressing and secreting intact, correctly assembled antibodies into the culture medium at such extraordinarily high levels. However, this does not contravene my opinion and experimental data refuting the enablement rejection because this unexpected result (the obtained yields of intact antibody) has been reproducibly achieved using diploidal yeast strains substantially produced according to the teachings of the subject application using several different fermentation techniques which those skilled in the art would have been aware of as of the date of the present invention. My opinion is further supported by the fact that these results have been reproducibly achieved with different antibody sequences, antibodies against different antigens, different diploidal yeast strains, and using different fermentation conditions.

8.) In providing my opinion, I reference experimental results obtained here at Alder and by Alder's collaborators which were obtained using fermentation methods that would have been well known to one of skill in the art as of October 22, 2003.

9.) I attach herewith as Appendices A-C graphical results obtained from experiments performed either at Alder Biopharmaceuticals, Inc., or under contract at a remote fermentation facility. In the case of the experiments conducted at Alder and at the University of British Columbia under contract to Alder, I supervised and/or directed these experiments. For the experiments performed by the Contract Manufacturing Organization (CMO), I was a key member of the Alder project team managing this contract research. In each of the

experiments in Appendices A-C, transformed diploid yeast produced according to the teachings of the '074 application were placed into fermentation conditions to produce a certain humanized antibody. For each of these experiments, the cells were separated from the supernatant at the conclusion of the fermentation run, and the antibody titer was determined using a High Performance Liquid Chromatograph (HPLC). I have summarized the results of the experiments below:

Appendix A - In this experiment, a diploid *Pichia pastoris* strain was created by the mating of two haploid strains. Both haploid strains were transformed prior to mating, one with a construct to express a heavy antibody chain and another to express a light antibody chain. Expression of both heavy and light chains was under the control of the GAP promoter. The resulting diploid *Pichia pastoris* strain was cultured for about 100 hours. These cells were cultured using an exponential feed technique to provide the fermentation media. The results demonstrate a final supernatant titer for antibody #1 of between about 450 to about 570 mg/L at about 90 hours fermentation time, and greater than 600 mg/L at 120 hours of fermentation time. The process "D8" was done in a 2000L fermentor in North Carolina by Alder's CMO. The A26 process was conducted at Alder Biopharmaceuticals in Applikon 15L stainless steel reactors. Finally, the B5 process was conducted at Alder Biopharmaceuticals using 10L New Brunswick glass vessel reactors.

Appendix B - In this experiment, several diploid *Pichia pastoris* yeast strains were built as mentioned in Appendix A. Three different strains expressing three different antibodies are compared using a similar process. The experiment "C2" expressing "Antibody 1" was conducted by Alder Biopharmaceuticals' collaborator at the University of British Columbia in a 1L glass vessel Applikon reactor, and the titers reached at about 90 hours of fermentation were approximately 650 mg/L. The experiments "L22" and "L66" expressing "Antibody 2" and "Antibody 3", respectively, were conducted in Alder's Fermentation Research facility and reached a secreted antibody titer between about 850 and about 1250 mg/L at about 90 hours of fermentation.

Appendix C – In this experiment, a diploid *Pichia pastoris* yeast strain built as mentioned in Appendix A was used. A cell bank was generated in April 2007 and tested in process "B6" within a week of the cell bank's generation. The same cell bank was used in August 2009 in two experiments identified as "L133" and "L134". "B6" was run in New Brunswick 10L glass vessels at Alder Biopharmaceuticals. "L133" and "L134" were run in 2.5L Labfors glass vessels at Alder Biopharmaceuticals. Even though the cell bank was greater than 2 years old, the results demonstrate comparable results. Therefore, diploid *Pichia pastoris* strains seem stable under common storage conditions (frozen in glycerol at -80°C) for greater than 2 years.

10.) On page 14, paragraph [64] of the '074 application, it is stated that "[h]ost cells are ... cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants or amplifying the genes encoding the desired sequences. A number of minimal media suitable for the growth of yeast are known in the art ... The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan."

11.) The media used to generate the results detailed in Appendices A, B and C, is comparable to the basic media described in paragraph [64] of the '074 application. The components of the media used to generate the results detailed in Appendices A, B and C is provided herewith in Appendix D. As someone skilled in the art would do, we optimized multiple parameters such as pH, temperature, concentration of divalent cations etc. *See, for example*, U.S. Patent No. 5,330,901, column 8, for carbon-energy sources and nutrients known to be useful for culturing *Pichia pastoris*. *See* Goodrick *et al*, Biotechnol Bioeng., 74 (6): 492-497 (2001), and U.S. Patent No. 5,440,018, column 11, regarding pH parameters for expression of proteins in *Pichia pastoris*. *See also* Sears *et al*, Yeast, 14:783-790 (1998), and Waterham *et al*, Gene, 196: 37-44 (1997) for exemplary carbon sources that may be used in conjunction with the GAP promoter. In addition, additives to improve titers were investigated and yeast extract was added. The addition of such additives is common in industry as described by Werten *et al*, Yeast, 15:1087 (1999), and in U.S. Patent No. 6,232,111.

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12.) As supported by reference citations identified herein, and further based on my knowledge in the relevant art (yeast fermentation), both the exponential feed technique and the step feed technique which were used to provide this media to the *Pichia pastoris* yeast cells during the fermentation are techniques that would have been well known to one of ordinary skill in the art as of October 22, 2003. *See* the following articles, disclosing the use of these techniques and their applicability to the fermentation of yeast and *Pichia pastoris* in particular published or submitted before October 22, 2003:

- Use of exponential feed strategy: Modeling *Pichia pastoris* Growth on Methanol and Optimizing the Production of a Recombinant Protein, The Heavy-Chain Fragment C of *Botulinum* Neurotoxin, Serotype A. Zhang *et al*, Biotechnol Bioeng., 70:1-8 (2000).

- Use of step feed strategies: Methods in Molecular Biology, volume 103, *Pichia* Protocols, edited by David R. Higgings and James M Cregg, chapter 9, page 117, and chapter 11, page 160 (1998).

Therefore, one skilled in the art would have been aware of several different methods that would have achieved this result. Of course with respect to my conclusions it would be generally understood by one skilled in the art that some amount of "optimization" may result in further enhanced expression levels. However, in my opinion this "optimization" would have been within the abilities of one of skilled in the art as of October 22, 2003, and would not rise to the level of "undue experimentation".

13.) Thus, in my opinion one of ordinary skill in the art following the teachings of the '074 application as of October 22, 2003, and using the well known techniques for delivery of feed to diploid *Pichia pastoris* yeast cells (which have been transformed to express and secrete into the culture medium a complete full length monoclonal antibody), would have been able, absent undue experimentation, to reproducibly achieve expression levels of at least 100 mg/l or higher in the fermentation supernatant following a reasonable fermentation time.

14.) I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

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these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the patent.

Executed on Sept 15 2009

Declarant's Signature: 

Leon Garcia-Martinez, Ph.D.

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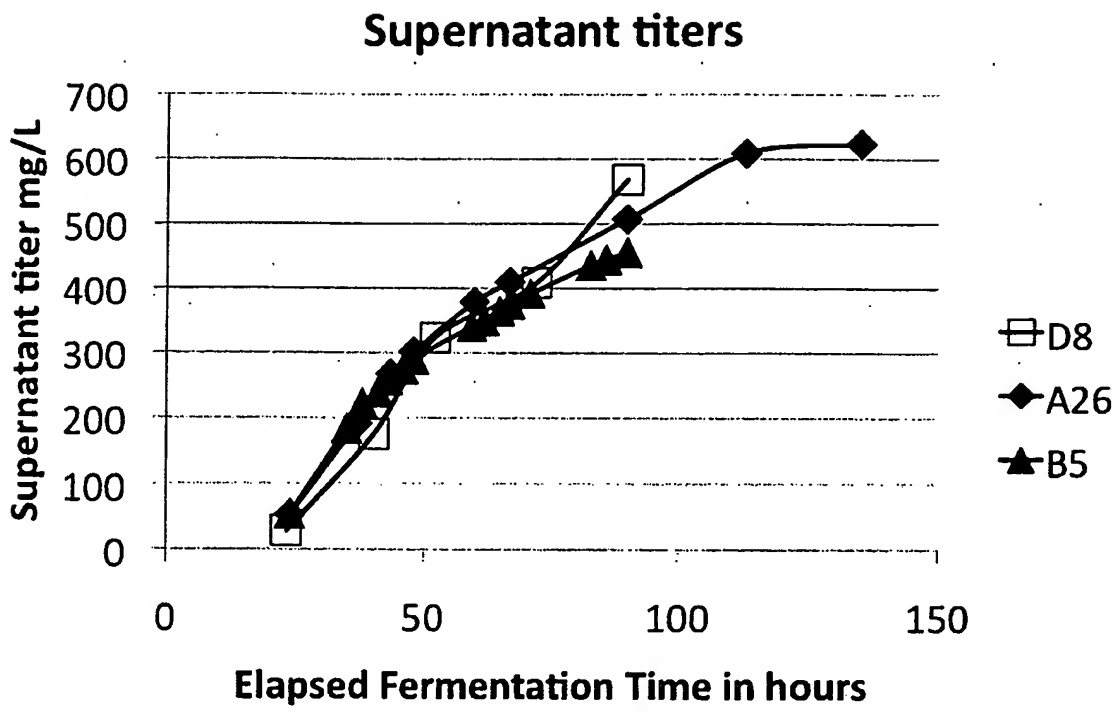
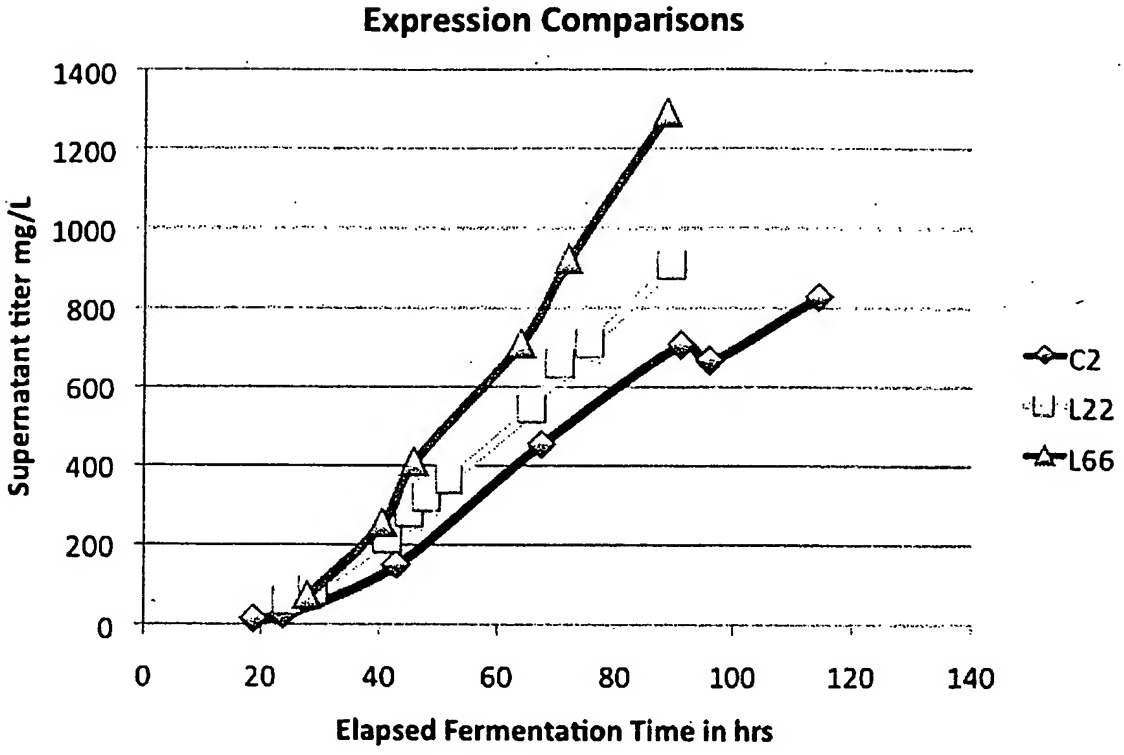
Woodinville, WA 98072

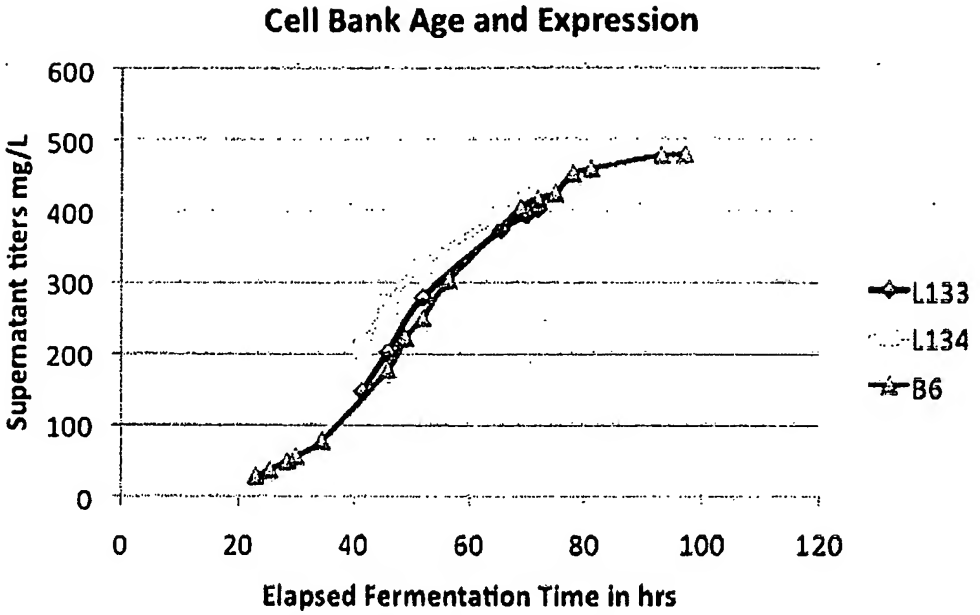
Appendix A

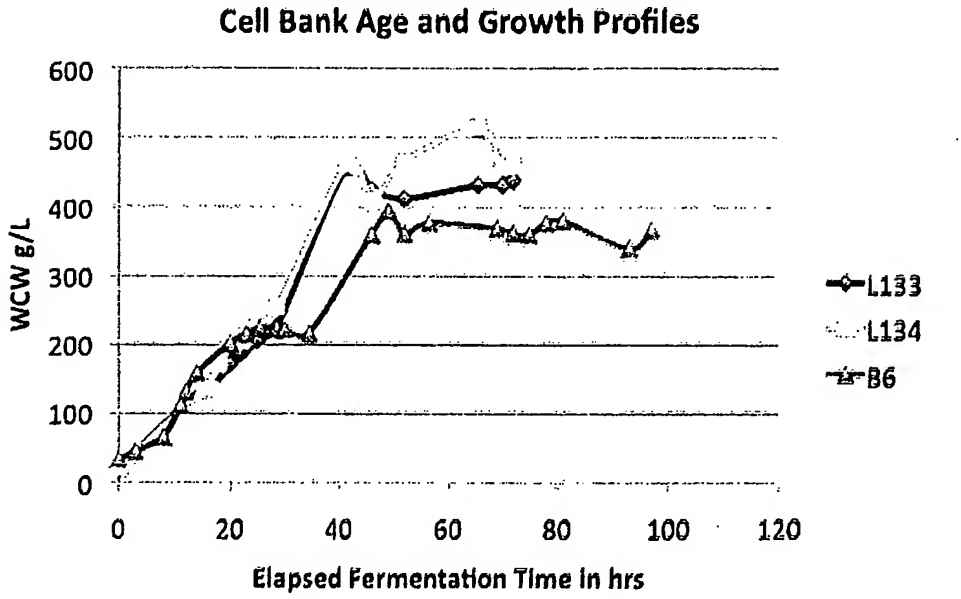

Supernatant titers
Supernatant titer mg/L
700
600
500
400
300
200
100
0
0
50
100
150
Elapsed Fermentation Time in hours
D8
A26
B5

Appendix B


Expression Comparisons
Supernatant titer mg/L
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
Elapsed Fermentation Time in hrs
C2
L22
L66

Appendix C


Cell Bank Age and Expression
Supernatant titers mg/L
600
500
400
300
200
100
0
0
20
40
60
80
100
120
Elapsed Fermentation Time in hrs
L133
L134
B6


Cell Bank Age and Growth Profiles
WCW g/L
600
500
400
300
200
100
0
0
20
40
60
80
100
120
Elapsed Fermentation Time in hrs
L133
L134
B6

Appendix D

Batch Medium Components
Glycerol or Glucose as carbon source
Yeast Extract
Potassium hydroxide
85% Phosphoric acid
Magnesium sulfate
Potassium sulfate
Ammonium sulfate
Sodium citrate
Ammonium Phosphate
Potassium Phosphate
PTM1
Antifoam 204

Feed Components
Glucose
Yeast Extract
PTM1
Potassium phosphate
Magnesium sulfate

PTM1 Components
Cupric sulfate
Sodium iodide
Manganese sulfate
Sodium molybdate
Boric acid
Cobalt chloride
Zinc chloride
Ferrous sulfate
Biotin
Sulfuric acid

The CV Appendix

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Director Antibody Therapeutics

Over twelve years of diverse biotech experience in multiple roles addressing aspects in the drug discovery process. Led a group of ten scientists (Ph.D. and M.S./B.S) in a program which led to the identification of a therapeutic antibody now in Phase II clinical trials. Supervised the technology development and implementation efforts that led to the expression of a full-length monoclonal antibody in *Pichia pastoris* at 2000L scale to support clinical trials. Led a target validation project that resulted in two patent applications (one published) and co-lead an second effort comparably resourced. Central scientist in a collaboration that developed, implemented and managed a high-throughput in vitro transcription screen for the discovery of small molecule inhibitors of HIV which resulted in a major milestone payment for the company. Broad experience with biochemical and cellular assay design and implementation, protein expression, purification and characterization.

APPOINTMENTS AND POSITIONS

2005 – Current Director Antibody Therapeutics

- Lead a team in the discovery of novel therapeutic antibody molecules. One molecule, ALD518 is currently in Phase IIb clinical trials.
- Instrumental on the development of MabXpress, a *Pichia pastoris* based technology for the expression of full-length monoclonal antibodies.
- Supervised a team for the development of technology for a *Pichia pastoris* fermentation process using diploid strains and the subsequent downstream purification of full length monoclonal antibodies. This technology was transferred to a CMO, further optimized and GMP Phase I and Phase IIa/b material was produced at the 2000L scale.
- Participated in reviewing and acceptance of batch records for two GMP manufacturing campaigns using *Pichia pastoris*.
- Participated as "person-in-plant" during multiple GMP manufacturing campaigns.

These efforts resulted in the production of the first full-length monoclonal antibody molecule in *Pichia pastoris* that has been tested in Humans.

2004 – 2005 Sr. Scientist. / Project Leader Icos Corporation

- Planned and led the execution of a target validation program for the adhesion molecule CD11c.
- Led a team of eight scientist and research associates.
- Cell-based characterization of antibody molecules that modulate cell-adhesion biology.
- Performed in-vivo evaluation of antibodies and toxin-conjugated antibody molecules.

Some of these efforts were published in Leukocyte Research in 2007

2002 – 2004 Project Leader / Sr. Scientist. Celltech R&D Inc.

- Planned and led the execution of a target validation program (immunology).
- Supervised two Associate Scientists (Sr. and level II) and led a team of 10 scientists and research associates in a matrix management environment.
- Expressed and purified antibodies and antigens (10s of µgs to 100mgs).
- Designed and validated cell-based and in vitro assays (e.g. novel MLRs, cell migration, Tg-specific response assays) .
- In vivo (mice) evaluation of lead therapeutic proteins (antibodies and recombinant protein agents).
- Initiated and managed external activities supporting the target validation effort.

- Reported project status and advised management both in the US and the UK regarding resource allocation for this and other projects.

These efforts resulted in two published applications, as well as a published manuscript and a second one in preparation. Participated in the triage and prioritization of targets regarding multiple research initiatives.

2001 – 2002 Sr. Scientist. Celltech R&D Inc.

- Designed, executed and supervised in vitro and cell based assay development, implementation and validation for two target validation projects (including cell proliferation, GTP-γ-S, chemotaxis, cellular differentiation, etc.) directed at neutrophil-driven and epidermal inflammation.
- Provided biochemical and cellular support for structure-activity relationship study for a medicinal chemistry program.
- Used organotypical cultures for testing of lead biologicals.
- Expressed and purified proteins in S2, baculo and bacteria systems.
- Generated and analyzed transgenic mice.
- Supervised two associate scientists (level I and Sr.).

The efforts resulted in intellectual property that is in patent prosecution.

1998 – 2000 Scientist II and Sr. Scientist. Signal Pharmaceuticals (now part of Celgene Corporation).

- Designed, implemented and supervised high-throughput screens looking for inhibitors of specific steps in viral life cycle (HIV and HCV).
- Responsible for the large scale (>100k compounds) screening campaign towards defined therapeutic targets.
- Responsible for protein expression, purification and quality control for these HTS efforts.
- Implemented laboratory automation.
- Coordinated efforts with chemistry for compound selection and prioritization.
- Reported project status and coordinated efforts with Dupont Pharmaceuticals as part of this collaboration.
- Supervised two associate scientists (level II and Sr.)

Optimization of this screen resulted in a 0.6% hit rate and 85% confirmation rate, that triggered the payment of a milestone for the company.

1993 – 1998 Instructor / Post-Doctoral Fellow. UT Southwestern Medical Center at Dallas. Laboratory of Dr. Richard B. Gaynor.

- Virology (HIV).
- Purified endogenous multi-protein complexes.
- Expressed and purified recombinant proteins (Bacteria, mammalian (vaccinia), baculo).
- Developed and validated several in vitro assays for protein functionality.
- Established several cell lines for protein production and analysis.
- Established key steps on the mechanism of transactivation by HIV Tat protein.
- Supervised two laboratory technicians.

Published nine papers in renowned peer reviewed journals.

1998 – 2000 Doctoral Student. UT Austin. Laboratory of Dr. Dean Appling.

- Determined the compartmentalization of purine biosynthesis to the mitochondria
- Conducted several subcellular fractionation studies.
- Purified and analyzed endogenous and recombinant proteins.
- Conducted Yeast (S. cerevisiae) genetic studies on purine biosynthesis.

These results were part of my Ph.D. Dissertation (Studies on the compartmentation of folate mediated metabolism). A part of this thesis was published in an internationally recognized peer reviewed journal.

1982 – 1986 Instituto Technologico de Monterrey (ITESM). Mexico.

- Prepared polar non polar and mixed plant extracts for subsequent fractionation.
- Separated secondary metabolites from higher plants using flash chromatography.
- Analyzed and determined the structure of several plant metabolites using thin layer chromatography, NMR, IR and UV spectropscopy.

Thesis: "Contribution to the chemical study of Senecio praecox and Salvia lasiantha".

EDUCATION

1988 – 1993 Ph. D. (Biochemistry), University of Texas, Austin, TX.

1982 – 1986 B. S. with Thesis (Chemistry), Instituto Tecnologico y de Estudios Superiores de Monterrey (ITESM), Monterrey N. L. Mexico.

Additional Training

2002 "Advance Course in Immunology" by the American Association of Immunologist at Standford University in California. An intensive course presenting recent advances in understanding of the biology of the immune system and its role in health and disease. Faculty composed of leading experts in their respective fields.

2001 "From the Laboratory to Leadership" a program offered through the Washington Biotechnology and Biomedical Association. Kirkland, WA. This unique program is custom-designed for executives and managers in scientific fields who must direct highly productive teams and prospering businesses in emerging scientific technologies.

2001 "REAL Project Management". Formal project management training with emphasis on development and implementation of project management skills.

HONORS AND AWARDS

Pediatric AIDS Foundation Scholar (1995).

Eakin Centennial Fellowship in Biochemistry (1993).

Professional Development Award from the Office of Graduate Studies,
University of Texas at Austin (1992).

GRANTS

Scholar Award, Pediatric AIDS Foundation (1995 - 1997).

INVITED LECTURES

A Robust Expression Platform for the Rapid Production of Full-Length Therapeutic Antibodies in *Pichia pastoris*. American Chemical Society, Biotechnology Division, Washington DC (2009)

Manufacture of Full-Length Therapeutic Antibodies in *Pichia Pastoris*: Development and Optimization. Recent Advances in Fermentation Technology, Florida (2007)

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Update on the Molecular Biology of the Infection by HIV. Continual Medical Education. Colegio Médico. Rioverde S.L.P. México (1994).

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MEETING PRESENTATIONS

LF. García-Martínez, Ou S-HI., Mavankal G. and Gaynor RB. Analysis of the Basal and Tat-Induced Transcription Levels for the HIV-1 and 2 LTR's. FASEB summer research conferences: Transcription regulation during cell growth differentiation and development (1994).

LF. García-Martínez and Appling DR. Characterization of the Mitochondrial Oxidation of the Third Carbon of Serine. FASEB summer research conferences: Folic acid, Vitamin B-12 and One-Carbon Metabolism (1992).

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BOOK CHAPTERS

Garcia-Martinez LF and Garcia JV Human immunodeficiency viruses: Molecular biology. In: Encyclopedia of Virology. A. Granoff and R. Webster editors (1999).

PUBLICATIONS

1) Sadhu, C., Ting H.J., Lipsky, K., Hensley, L., **Garcia-Martinez, L.F.**, Simon, S.I., Staunton, D.E. CD11c/CD18: novel ligands and a role in delayed-type hypersensitivity. J. Leukoc. Biol. 81: 1395-1403 (2007).

2) **Garcia-Martinez LF,** Appleby MW, Staehling-Hampton K, Andrews DM, Chen Y., McEuen M, Tang P, Rhinehart RL, Proll S, Paeper B, Brunkow ME, Grandea AG 3rd, Howard ED, Walker DE, Charmley P, Johas M, Shaw S, Latham JA, Ramsdell F. A novel mutation in CD83 results in the development of a unique population of CD4+ T cells. J Imunol. 173 2995-3001 (2004).

3) **Garcia-Martinez LF**, Bilter GK, Wu J,O'Neill J, Barbosa MS and Kovelman R. In vitro high-throughput assay for modulators of transcrition. Anal. Biochem. 301 103-110 (2002).

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5) Liu L, Kwak YT, Bex F, **Garcia-Martinez LF**, Li XH, Meek K., Lane WS, Gaynor RB. DNA-dependent protein kinase phosphorylation of IkappaB alpha and IkappaB beta regulates NF-kappaB DNA binding properties. Mol. Cell Biol. 18 4221-4234 (1998).

6) **García-Martínez LF.**, Mavankal G., Neveu JM., Lane SW., Ivanov D. and Gaynor RB. Purification of a Tat-Associated Kinase Reveals a TFIIH Complex that Modulates HIV-1 EMBO J. 16 2836-2850 (1997).

7) Harrich D., Ulich C., **García-Martínez LF**. and Gaynor RB. Tat is Required is Required for Efficient HIV-1 Reverse Transcription EMBO J. 16 1224-1235 (1997).

8) **García-Martínez LF.**, Ivanov D. and Gaynor RB. Association of Tat with Purified HIV-1 and HIV-2 Transcription Preinitiation Complexes J. Biol. Chem. 272 6951-6958 (1997).

9) Orsini MJ., **García-Martínez LF.**, Mavankal G., Gaynor RB and Debouck CM. Purification and Functional Characterization of Wild-Type and Mutant HIV-1 and HIV-2 Proteins Expressed in Escherichia coli. Protein Expression & Purification 8 238-246 (1996).

10) **LF. García-Martínez**, Mavankal G., Peters P., Wu-Baer F. and Gaynor RB. Tat Functions to Stimulate the Elongation Properties of Transcription Complexes Paused by the Duplicated TAR RNA Element of Human Immunodeficiency Virus 2. J. Mol. Biol. 254 350-363 (1995).

11) S-H I.Ou, Wu F., Harrich D., **García-Martínez LF**. and Gaynor RB. Cloning and Characterization of a Novel Cellular Protein, TDP-43, That Binds to Human Immunodeficiency Virus Type 1 TAR DNA Sequence Motifs. J. Virology 69 3584-3596 (1995).

12) S-H I.Ou, **García-Martínez LF.**, Paulsen EP. and Gaynor RB. Role of Flanking E Box Motifs on HIV-1 TATA Element Function. J. Virology 68 7188-7199 (1994).

13) **LF. García-Martínez** and Appling DR. Characterization of the Folate-Dependent Mitochondrial Oxidation of Carbon Three of Serine. Biochemistry 32 4671-4676 (1993).

PATENT APPLICATIONS

Olson, Katie; Dutzar, Ben; Jensen, Anne; **Garcia-Martinez,** Leon; Ojala, Ethan; Latham, John. "Antibodies to TNF alpha and use thereof" 20090022659.

Garcia-Martinez; Leon, Jensen, Anne; Olson, Katie; Dutzar, Ben; Ojala, Ethan; Kovacevich, Brian; Latham, John; Smith, Jeffrey. "Antibodies to IL-6 and the use thereof" 20090028784.

Leon Fernando Garcia Martinez, Karen Staehling-Hampton, Fred Ramsdell, Mark Appleby and Sean Proll "Manipulation of Cytokine Levels using CD83 Gene Products" WO03045318 (2003).

Garcia-Martinez; Leon Fernando; Chen, Yuching; Andrews, Dawn; "Modulating immune responses" 20080299122.

U.S. Patent No. 5,330,901, to Prevatt *et al.*

US005330901A

United States Patent [19]

Prevatt et al.

[11] Patent Number: 5,330,901

[45] Date of Patent: Jul. 19, 1994

[54] EXPRESSION OF HUMAN SERUM ALBUMIN IN *PICHIA PASTORIS*

[75] Inventors: William D. Prevatt; Kotikanyadan Sreekrishna, both of Bartlesville, Okla.

[73] Assignee: Research Corporation Technologies, Inc., Tucson, Ariz.

[21] Appl. No.: 691,079

[22] Filed: Apr. 25, 1991

[51] Int. Cl.[5] C12P 21/02; C12N 1/19

[52] U.S. Cl. 435/69.6; 435/254.23; 935/37

[58] Field of Search 435/69.1, 69.6, 71.1, 435/172.1, 183, 252.3, 320.1, 254.23; 935/22, 28, 33, 37, 66, 69

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4,775,622	10/1988	Hitzeman	435/69.4
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Primary Examiner—Richard A. Schwartz

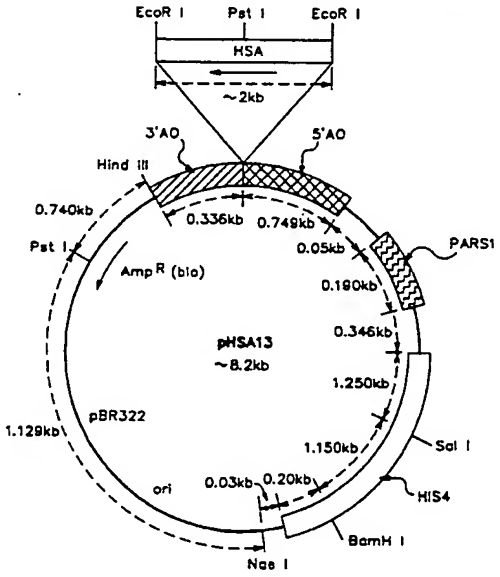
Assistant Examiner—J. Ketter

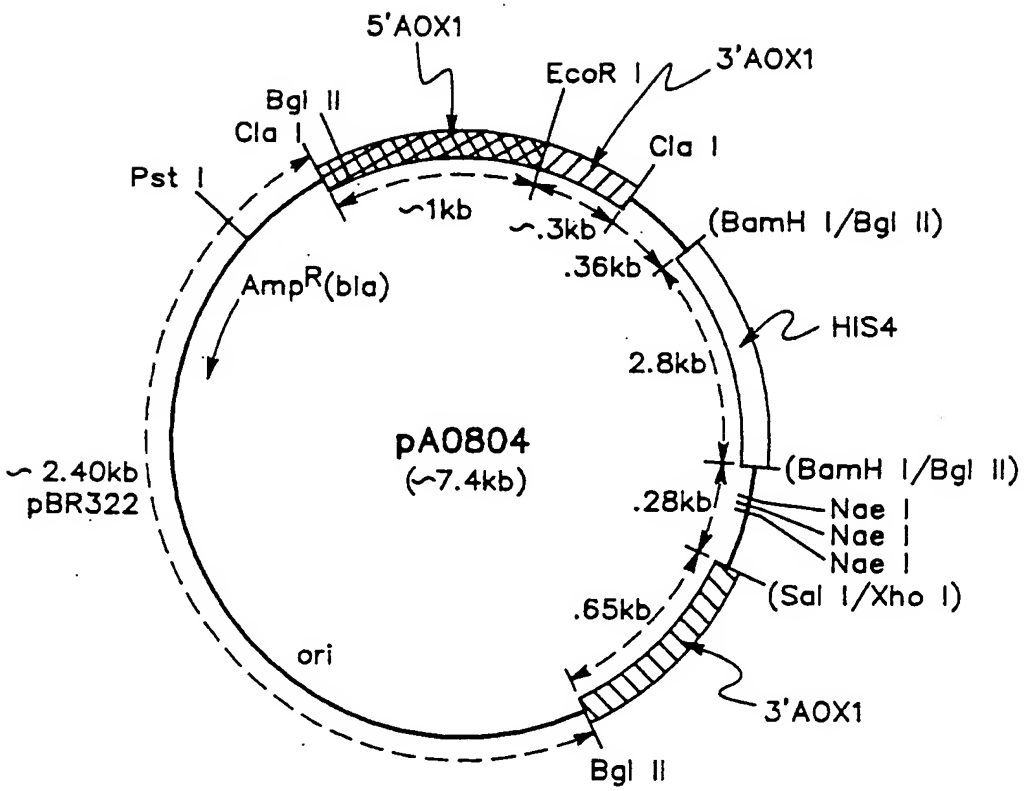
Attorney, Agent, or Firm—Scully, Scott, Murphy & Presser

[57] ABSTRACT

A process for the production of HSA in *Pichia pastoris* cells comprising cultivating *Pichia pastoris* cells capable of expressing HSA at a pH of about 5.7 to about 6.4 contemporaneously with the expression of HSA.

16 Claims, 6 Drawing Sheets

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3'AOX1
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ori
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Bgl II

FIG. 1

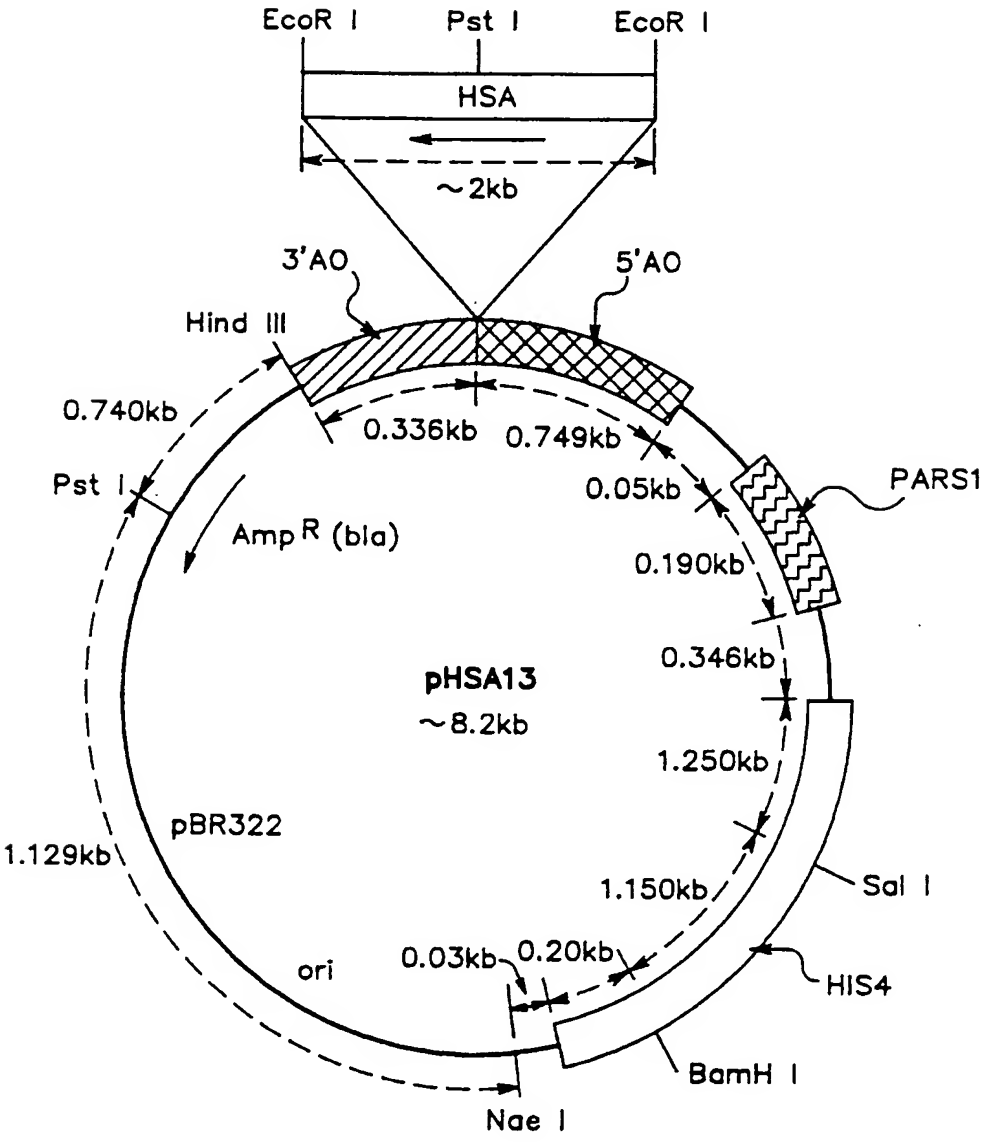
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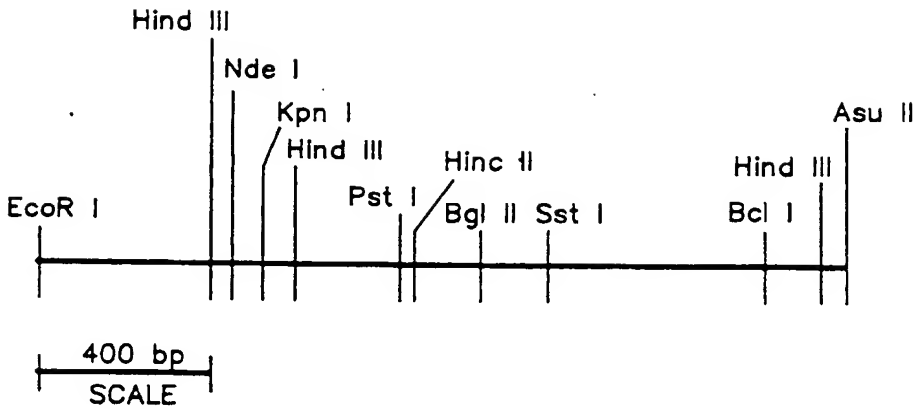
FIG. 2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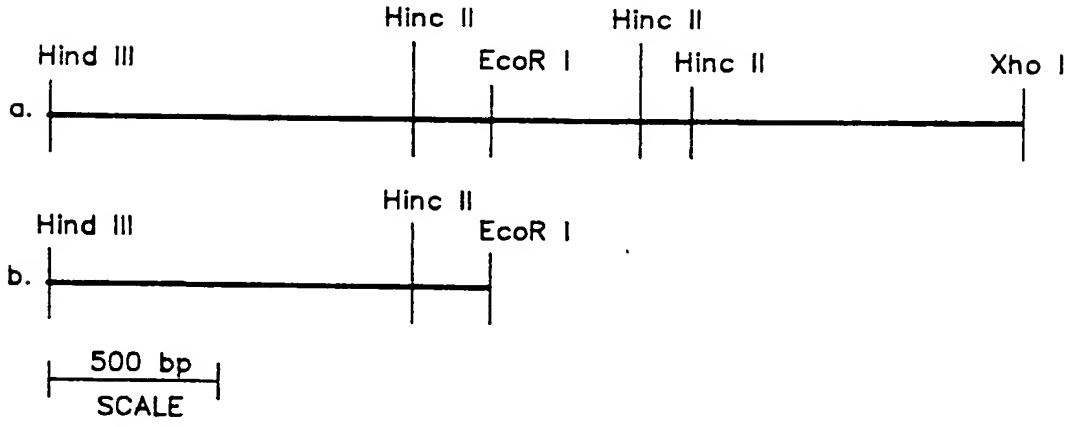
SCALE

FIG. 3


Hinc II
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b.
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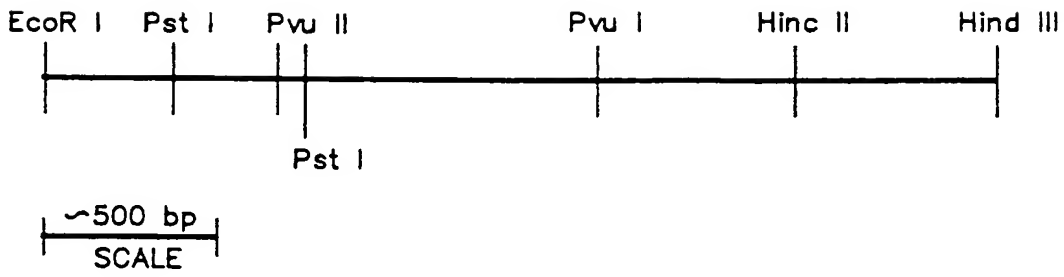
FIG. 4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Hinc II
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FIG. 5

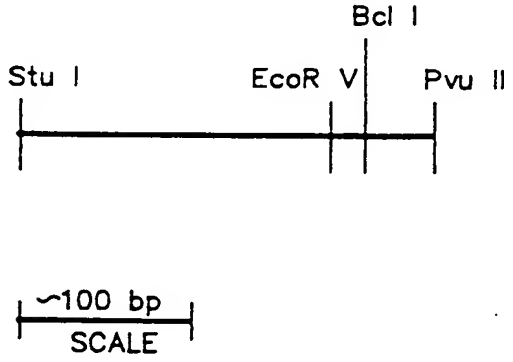
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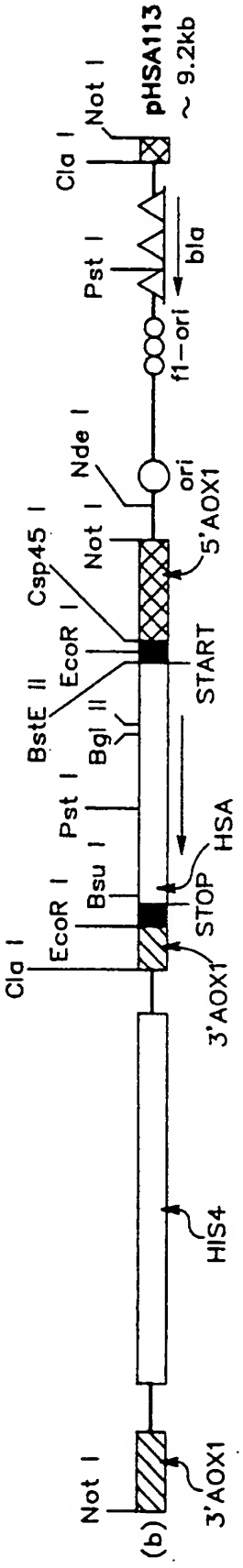
FIG. 6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Nde I
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EcoR I
BstE II
START
Bgl II
Pst I
HSA
Bsu I
STOP
EcoR I
Cla I
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HIS4
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(b)
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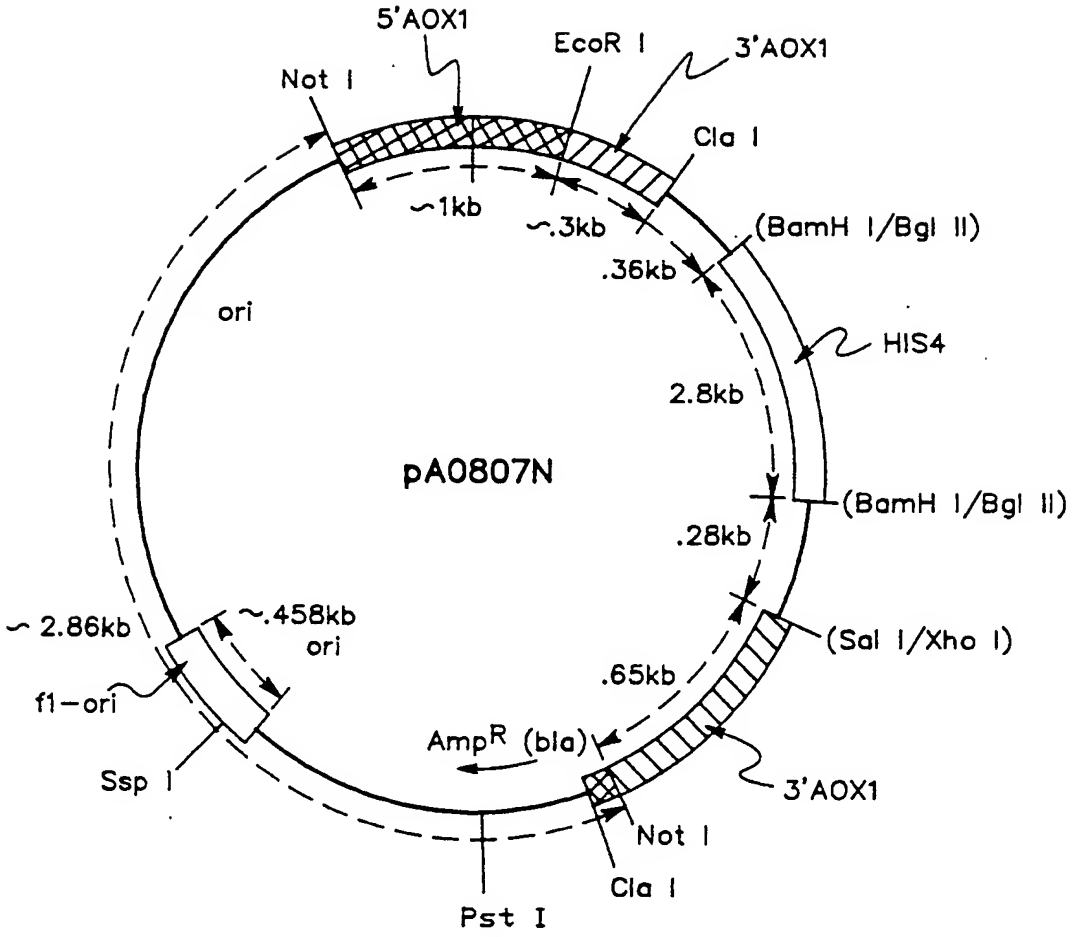
1kb

FIG. 7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~.458kb
ori
(Sal I/Xho I)
.65kb
f1-ori
AmpR (bla)
Ssp I
3'AOX1
Not I
Cla I
Pst I

FIG. 8

EXPRESSION OF HUMAN SERUM ALBUMIN IN *PICHIA PASTORIS*

FIELD OF THE INVENTION

This invention relates to the field of recombinant DNA biotechnology. In one aspect, this invention relates to a process for the improved expression of secreted human serium albumin (HSA) in *Pichia pastoris*.

BACKGROUND

Human serum albumin is the most abundant plasma protein of adults. The concentration of albumin is 40 mg/ml, or 160 g of albumin circulating throughout the human body for a 70 Kg adult male. This protein maintains osmotic pressure and functions in the binding and transport of copper, nickel, calcium (weakly, at 2-3 binding sites), bilirubin and protoporphyrin, long-chain fatty acids, prostaglandins, steroid hormones (weak binding with these hormones promotes their transfer across the membranes), thyroxine, triiodothyronine, crystine, and glutathione. According to Peters, T. and Reed, R. G. in *Albumin: Structure, Biosynthesis and Function*, (Peter, T. and Sjoholm, J. eds.) 1977 p. 11-20, over 10,000 kilograms of purified albumin are administered annually in the United States alone to patients with circulatory failure or with albumin depletion.

Currently the only commercial source of HSA is from fractionated blood. Considering the possible dangers of blood borne contaminants and pathogens, it would be a considerable contribution to the commercial production of HSA to develop alternate methods of producing HSA. With the advent of recombinant DNA technology, it is now possible to produce HSA by alternate methods.

HSA has also been expressed in *Saccharomyces cerevisiae* as disclosed by Etcheverry et al. in *Bio/technology*, August 1986, p. 726 and Arjum Singh in EPA 123,544. Etcheverry disclosed HSA expression intracellularly in a concentration of approximately 6 mg/l and the secretion of HSA which remained cell associated. Arjum Singh also disclosed the expression of HSA in Saccharomyces cerevisiae in combination with the α-factor promoter and signal sequence. Singh appears to have been able to achieve an intracellular production level of approximately 25 mg/l and a secreted production level of 3 mg/l. *Pichia pastoris* has also been used to express HSA as is disclosed in EPA 344,459. The concentration of HSA produced in *Pichia pastoris* appears to be about 89 ng HSA/mg of protein. Although the process for producing HSA in recombinant expression system has been established by these experiments it would be desirable to optimize these processes to achieve the maximum possible HSA production.

Therefore, it would be a significant contribution to the art to provide a process for increasing the yeild of HSA from the recombinant expression of HSA in microorganism such as *Pichia pastoris*.

Therefore, it is an object of this invention to provide a process for increasing the yield of HSA produced in a recombinant expression systems.

SUMMARY OF THE INVENTION

In accordance, we have discovered a process for improving the secreted expression of HSA in *Pichia pastoris* cells comprising:

(a) cultivating in a fermentation broth transformed *Pichia pastoris* cells capable of expressing HSA under conditions suitable for the sustained viability of said *Pichia pastoris* cells under suitable conditions for the expression of HSA by said *Pichia pastoris* cells; and maintaining the pH of said fermentation broth from a pH of from about 5.7 to about 6.0 contemporaneously with the expression of HSA.

DETAILED DESCRIPTION OF THE FIGURES

FIG. 1 provides a representation of plasmid pA0804 which contains a linear site-specific integrative vector in the fragment clockwise from BglII to BglII. The structural gene may be inserted in the unique EcoRI site of this plasmid. This plasmid may be recovered from the plasmid DNA of NRRL B-18114 by EcoRI digest and gel electrophoresis to recover a linear ~7.4 kb EcoRI fragment corresponding to FIG. 1.

FIG. 2 provides a representation of pHSA13 in circular form.

FIG. 3 provides a restriction map of the AOX1 5' regulatory region isolated from *Pichia pastoris*.

FIG. 4 provides a restriction map of the DAS1 5' regulatory region isolated from *Pichia pastoris*.

FIG. 5 provides a restriction map of the AOX1 3' termination sequence isolated from *Pichia pastoris*.

FIG. 6 provides a restriction map of the DAS1 3' termination sequence isolated from *Pichia pastoris*.

FIG. 7 provides a representation of pHSA113 in linear form.

FIG. 8 provides a representation of plasmid pAO807N which contains a linear site-specific integrative vector in the fragment clockwise from NotI to NotI. The structural gene may be inserted in the unique EcoRI site of this plasmid.

DETAILED DESCRIPTION

Generally *Pichia pastoris* is optimally grown at from about pH 4.8 to about pH 5.2. Between this pH range *Pichia pastoris* provided with a suitable nutrient media exhibits robust growth. This pH range also appears to result in high levels of expression of several foreign proteins such as hepatitis B surface antigen. This pH range also appeared to provide high levels of expression with human serum albumin (HSA). For example growing *Pichia pastoris* cells which had been transformed with a vector containing a HSA structural gene operably linked to a 5' regulatory region (i.e. a promoter) and a 3' termination sequence, the expression levels of HSA which had been obtained were approximately 0.71 to 0.81 grams/liters of HSA in the fermentation broth. However, we have been able to further increase this yield by at least 50% by taking the unprecedented step of shifting the pH of the fermentation broth from about 5.2 to in the range of from about pH 5.7 to about pH 6.4, with a preferred pH range of from about pH 5.7 to about pH 6.0 and most preferably a pH in the range of from pH 5.75 to pH 5.85. The increased secretion levels obtained in the upper limits of the pH range (i.e. from in the range of pH 6.0 to pH 6.4) have been confirmed in shake tube optimization studies which indicate that the presence of yeast extract and peptone together with aeration will provide optimal HSA secretion in shake tubes. However, the use of yeast extract, peptone and excess aeration is not believed necessary in large scale fermentation where the pH can be continuously monitored. We believe that this higher pH level will increase the yield of any *Pichia pastoris* strain transformed with an expression cassette containing a promoter and a

structural gene encoding a signal sequence and the mature HSA protein. Further it would appear that this result will be applicable to a variety of heterologous structural genes which encode a signal sequence and a mature heterologous protein. Suitable heterologous proteins which may be expressed at higher levels utilizing this method include but are not limited to heterologous proteins selected from the group consisting of tissue plasminogen activator, albumins (such as human serum albumin), lysozymes (such as bovine lysozyme), interferons (such as gamma-interferon and beta-interferon) and invertase. Each of the heterologous structural genes utilized in the present invention must have a signal sequence operably linked to the 5' end of sequence coding for the mature heterologous protein to effect the secretion of the mature protein. For example the tissue plasmigen activator, human serum albumins, bovine lysozyme, beta-interferon, gamma-interferon and invertase proteins may all be secreted utilizing the native signal sequence. Furthermore these proteins may also be secreted utilizing secretion signal sequences from *Pichia pastoris* such as the acid phosphatase signal sequence disclosed in U.S. patent application Ser. No. 07/627,539 filed Dec. 14, 1990 by Richard Buckholz assigned to Phillips Petroleum Company (incorporated herein by reference) or the alpha-mating factor signal sequence from *Saccharomyces cerevisiae.*

Utilizing the present invention, HSA secretion levels of approximately 1-3 grams of authentic HSA per liter of fermentation broth have been obtained. This invention thus provides a means for the high level secretion of HSA. Achieving these levels of HSA production is a significant advancement over the prior production levels, since at the level of 1-3 grams per liter the recovery of HSA in high yields with high purities is possible.

To express the HSA structural gene, the gene must be operably linked to a 5' regulatory region and a 3' termination sequence, which forms an expression cassette which will be inserted into a host (usually a microorganism) via a vector (such as a plasmid or linear site-specific integrative vector). Operably linked as used in this context refers to a juxtaposition wherein the 5' regulatory region, structural gene, and 3' termination sequence are linked and configured so as to perform their normal function. 5' regulatory region or promoter as used herein means DNA sequences which respond to various stimuli and provide enhanced rates of mRNA transcription. 3' termination sequence are sequences 3' to the stop codon of a structural gene which function to stabilize the mRNA transcription product of the gene to which the sequence is operably linked (such as sequences which elicit polyadenylation). For the practice of this invention, it is preferred that the ATG of the structural gene be linked with as few intervening deoxyribonucleotides as possible to the 3' end of the 5' regulatory region, preferrably about 11 or less deoxyribonucleotides and most preferably 8 or less deoxyribonucleotides. It is also preferred that the adenine and thymine content of the intervening deoxyribonucleotides be in the range of from about 55 percent to about 64 percent. Further, it appears that there are nucleotide preferences for certain specific locations. Counting left from the ATG condon of the structural gene with the first position left being the −1 position, it appears that adenine or cytosine is the most preferred deoxyribonucleotide, in the −2 position the most preferred deoxyribonucleotide is either adenine or thymine, in the −3 position the most preferred deoxyribonucleotide is adenine or thymine and the most preferred nucleotide at the −4 position is adenine, thymine or cytosine. Currently, it is preferred that the AOX1 or DAS1 5' regulatory region having the restriction maps of FIGS. 3 and 4 or, the sequences provided as SEQ ID No: 1 and SEQ ID No: 2, respectively, be linked at their 3' end of the sequence to the ATG start codon of the HSA structural gene. One example of an appropriate linkages for the AOX1 5' regulatory region is illustrated below:

TABLE I

Construct Designation	End of the 5' Regulatory Region for AOX 1	Deoxyribonucleotide intervening before ATG start condon
pHSA413	5' - TTCGAAACG	5' - NONE

Several 5' regulatory regions have been characterized and can be employed in conjunction with the expression of HSA in *Pichia pastoris.* Exemplary 5' regulatory regions are the primary alcohol oxidase (AOX1), dihydroxyacetone synthase (DAS1), glyceraldehyde-3-phosphate dehydrogenase gene (GAP), acid phosphatase gene (PHO1) and the p40 regulatory regions, derived from *Pichia pastoris* and the like. The AOX1 5' regulatory region, DAS1 5' regulatory region and p40 5' regulatory region are described in U.S. Pat. No. 4,855,231, incorporated herein by reference. The GAP 5' regulatory region is disclosed in EPA 374,913 published Jun. 27, 1990, incorporated herein by reference. The PHO1 5' regulatory region is disclosed in U.S. patent application 07/672,539 filed Dec. 14, 1990, assigned to Phillips Petroleum Company. The presently preferred 5' regulatory regions employed in the practice of this invention are those characterized by their ability to respond to methanol-containing media, such regulatory regions selected from the group consisting of AOX1, and DAS1. The most preferred 5' regulatory region for the practice of this invention is the AOX1 5' regulatory region.

3' termination sequences should be utilized in the expression cassette as discussed above. 3' termination sequences may function to terminate, polyadenylate and/or stabilize the messenger RNA coded for by the structural gene when operably linked to a gene, but the particular 3' termination sequence is not believed to be critical to the practice of the present invention. A few examples of illustrative sources for 3' termination sequences for the practice of this invention include but are not limited to the *Hansenula polymorpha* and *Pichia pastoris* 3' termination sequences. Preferred are those derived from *Pichia pastoris* such as those selected from the group consisting of the 3' termination sequences of AOX1 gene, DAS1 gene, p40 gene GAP gene, PHO1 gene and HIS4 gene. Particularly preferred is the 3' termination sequence of the AOX1 gene.

Pichia pastoris may be transformed with a variety of HSA structural genes (in the inventive transformants discussed herein the HSA structural gene encodes both a signal sequence and a mature HSA protein). HSA structural genes have been sequenced by Lawn et al. *Nuc. Acids Res.* 9:6105 (1981), and Dugaiczyk et al., *Proc. Natl. Acad. Sci.* USA 79:71 (1982). These genes may also be obtained by reisolation of the genes by the technique of Lawn et al., Dugaiczyk et al. or synthesized in vitro by a custom gene manufacturer such as British Biotechnology, Ltd. One possible method of obtaining a HSA gene would be to screen a human liver cDNA library with oligonucleotide probes or screen a

human liver cDNA expression library with anti-HSA antisera to identify HSA expressing cDNAs. One suitable HSA structural gene is provided in SEQ ID NO: 3. Once a structural gene for HSA is recovered, it may be necessary to further tailor the gene. Following the isolation of an HSA structural gene, the gene is inserted into a suitable *Pichia pastoris* vector such as a plasmid or linear site-specific integrative vector.

Plasmid-type vectors have long been one of the basic elements employed in recombinant DNA technology. Plasmids are circular extra-chromosomal double-stranded DNA found in microorganisms. Plasmids have been found to occur in single or multiple copies per cell. Included in plasmid DNA is the information required for plasmid reproduction, e.g. an autonomous replication sequence such as those disclosed by James M. Cregg in U.S. Pat. No. 4,837,148, issued Jun. 6, 1989, incorporated herein by reference. Additionally one or more means of phenotypically selecting the plasmid in transformed cells may also be included in the information encoded in the plasmid.

Suitable integrative vectors for the practice of the present invention are the linear site-specific integrative vectors described by James M. Cregg, in U.S. Pat. NO. 4,882,279, issued Nov. 21, 1989, which is incorporated herein by reference. These vectors comprise a serially arranged sequence of at least 1) a first insertable DNA fragment; 2) a selectable marker gene; and 3) a second insertable DNA fragment. An expression cassette containing a heterologous structural gene is inserted in this vector between the first and second insertable DNA fragments either before or after the marker gene. Alternatively, an expression cassette can be formed in situ if a regulatory region or promoter is contained within one of the insertable fragments to which the structural gene may be operably linked.

The first and second insertable DNA fragments are each at least about 200 nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be transformed. The various components of the integrative vector are serially arranged forming a linear fragment of DNA such that the expression cassette and the selectable marker gene are positioned between the 3' end of the first insertable DNA fragment and the 5' end of the second insertable DNA fragment. The first and second insertable DNA fragments are oriented with respect to one another in the serially arranged linear fragment as they are oriented in the parent genome.

Nucleotide sequences useful as the first and second insertable DNA fragments are nucleotide sequences which are homologous with separate portions of the native genomic site at which genomic modification is to occur. For example, if genomic modification is to occur at the locus of the alcohol oxidase gene, the first and second insertable DNA fragments employed would be homologous to separate portions of the alcohol oxidase gene locus. Examples of nucleotide sequences which could be used as first and second insertable DNA fragments are deoxyribonucleotide sequences selected from the group consisting of the *Pichia pastoris* alcohol oxidase (AOX1) gene, dihydroxyacetone synthase (DAS1) gene, p40 gene, glyceraldehyde-3-phosphate dehydrogenase (GAP) gene, acid phosphatase (PHO1) gene and HIS4 gene. The AOX1 gene, DAS1 gene, p40 gene and HIS4 genes are disclosed in U.S. Pat. Nos. 4,855,231 and 4,885,242 both incorporated herein by reference. The designation DAS1 is equivalent to the DAS designation originally used in U.S. Pat. Nos. 4,855,231 and 4,885,242. The GAP gene is disclosed in EPA 374,913 published Jun. 27, 1990 incorporated herein by reference. The PHO1 gene is disclosed in U.S. patent application Ser. No. 07/627,539 filed Dec. 14, 1990, now U.S. Pat. No. 5,268,273, assigned to Phillips Petroleum Company, incorporated herein by reference.

The first insertable DNA fragment may contain an operable regulatory region which may comprise the regulatory region utilized in the expression cassette. The use of the first insertable DNA fragment as the regulatory region for an expression cassette is a preferred embodiment of this invention. FIG. 1 provides a diagram of a vector utilizing the first insertable DNA fragment as a regulatory region for a cassette. Optionally, as shown in FIG. 1, an insertion site or sites and a 3' termination sequence may be placed immediately 3' to the first insertable DNA fragment. This conformation of the linear site-specific integrative vector has the additional advantage of providing a ready site for insertion of a structural gene without necessitating the separate addition of a compatible 3' termination sequence.

If the first insertable DNA fragment does not contain a regulatory region, a suitable regulatory region will need to be inserted linked to the structural gene, in order to provide an operable expression cassette. Similarly, if no 3' termination sequence is provided at the insertion site to complete the expression cassette, a 3' termination sequence can be operably linked to the 3' end of the structural gene.

It is also highly desirable to include at least one selectable marker gene in the DNA used to transform the host strain. This facilitates selection and isolation of those organisms which have incorporated the transforming DNA. The marker gene confers a phenotypic trait to the transformed organism which the host did not have, e.g. restoration of the ability to produce a specific amino acid where the untransformed host strain has a defect in the specific amino acid biosynthetic pathway, or provides resistance to antibiotics and the like. Exemplary selectable marker genes may be selected from the group consisting of the HIS4 gene (disclosed in U.S. Pat. No. 4,885,242) and the ARG4 gene (disclosed in U.S. Pat. No. 4,818,700 incorporated herein by reference) from *Pichia pastoris* and *Saccharomyces cerevisiae*, the invertase gene (SUC2) (disclosed in U.S. Pat. No. 4,857,467 incorporated herein by reference) from *Saccharomyces cerevisiae*, or the $G418^R$/kanamycin resistance gene from the *E. coli* transposable elements Tn601 or Tn903.

Those skilled in the art recognize that additional DNA sequences can also be incorporated into the vectors employed in the practice of the present invention, such as, for example, bacterial plasmid DNA, bacteriophage DNA, and the like. Such sequences enable the amplification and maintenance of these vectors in bacterial hosts.

The insertion of the HSA structural gene into suitable vectors may be accomplished by any suitable technique which cleaves the chosen vector at an appropriate site or sites and results in at least one operable expression cassette containing the HSA structural gene being present in the vector. Ligation of the HSA structural gene may be accomplished by any appropriate ligation technique such as utilizing T4 DNA ligase.

The initial selection, propagation, and optional amplification of the ligation mixture of the HSA structural gene and a vector is preferably performed by transform-

ing the mixture into a bacterial host such as *E. coli* (although the ligation mixture could be transformed directly into a yeast host but, the transformation rate would be extremely low). Suitable transformation techniques for *E. coli* are well known in the art. Additionally, selection markers and bacterial origins of replication necessary for the maintenance of a vector in a bacterial host are also well known in the art. The isolation and/or purification of the desired plasmid containing the HSA structural gene in an expression system may be accomplished by any suitable means for the separation of plasmid DNA from the host DNA. Similarly the vectors formed by ligation may be tested, preferably after propagation, to verify the presence of the HSA gene and its operable linkage to a regulatory region and a 3' termination sequence. This may be accomplished by a variety of techniques including but not limited to endonuclease digestion, gel electrophoresis, or Southern hybridization.

Transformation of plasmids or linear vectors into yeast hosts may be accomplished by suitable transformation techniques including but not limited to those taught by Cregg and Barringer, U.S. Pat. No. 4,929,555; Hinnen et al., *Proc. Natl. Acad. Sci.* 75, (1978) 1929; Ito et al., *J. Bacteriol.* 153, (1983) 163; Cregg et al. *Mol. Cell Biol.* 5 (1985), pg. 3376; D. W. Stroman et al., U.S. Pat. No. 4,879,231, issued Nov. 7, 1989; or Sreekrishna et al., *Gene,* 59 (1987), pg. 115. Preferable for the practice of this invention is the transformation technique of Cregg et al. (1985). It is desirable for the practice of this invention to utilize an excess of linear vectors and select for multiple insertions by Southern hybridization.

The yeast host for transformation may be any suitable methylotrophic yeast. Suitable methylotrophic yeasts include but are not limited to yeast capable of growth on methanol selected from the group consisting of the genera Hansenula and Pichia. A list of specific species which are exemplary of this class of yeasts may be found in C. Anthony, *The Biochemistry of Methylotrophs,* 269 (1982). Presently preferred are methylotrophic yeasts of the genus Pichia such as the auxotrophic *Pichia pastoris* GS115 (NRRL Y-15851); *Pichia pastoris* GS190 (NRRL Y-18014) disclosed in U.S. Pat. No. 4,818,700; and *Pichia pastoris* PPF1 (NRRL Y-18017) disclosed in U.S. Pat. No. 4,812,405. Auxotrophic *Pichia pastoris* strains are also advantageous to the practice of this invention for their ease of selection. It is recognized that wild type *Pichia pastoris* strains (such as NRRL Y-11430 and NRRL Y-11431) may be employed with equal success if a suitable transforming marker gene is selected, such as the use of SUC2 to transform *Pichia pastoris* to a strain capable of growth on sucrose or an antibiotic resistance marker is employed, such as G418.

Transformed *Pichia pastoris* cells can be selected for by using appropriate techniques including but not limited to culturing previously auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype ("methanol slow"), or culturing in the presence of an antibiotic which is toxic to the yeast in the absence of a resistance gene contained in the transformant.

Isolated transformed *Pichia pastoris* cells are cultured by appropriate fermentation techniques such as shake flask fermentation, high density fermentation or the technique disclosed by Cregg et al. in, *High-Level Expression and Efficient Assembly of Hepatitis B Surface Antigen in the Methylotrophic Yeast, Pichia Pastoris* 5 Bio/Technology 479 (1987). Isolates may be screened by assaying for HSA production to identify those isolates with the highest HSA production level.

The cultivation of transformed *Pichia pastoris* can be conducted in an aqueous continuous or batch-fed manner, utilizing a variety of carbon-energy sources and/or nutrient sources. For the practice of the present invention, batch-fed fermentation is preferred. Suitable carbon-energy sources for growing *Pichia pastoris* include but are not limited to the carbon-energy source selected from the group consisting of methanol, glycerol, sorbitol, glucose, fructose and combinations of any two or more thereof. Preferred carbon-energy sources for growing *Pichia pastoris* are carbon-energy sources selected from the group consisting of methanol, glycerol, and combinations thereof. A suitable nutrient source or media for *Pichia pastoris* would include at least one nitrogen source, at least one phosphate source, at least one source of minerals such as iron, copper, zinc, magnesium, manganese, calcium, and other trace elements, and vitamins (such as biotin, pantothenic acid, and thiamine as required).

Suitable sources of at least one carbon-energy source and nutrients can be obtained from a variety of sources or may consist of a single source. However, preferred are at least one carbon-energy source and/or nutrient sources which have a defined character. One carbon-energy source and/or nutrient composition which has proven effective is:

TABLE II

Carbon-Energy Source and Nutrients Component per Liter of Water	
Carbon-energy Source (glycerol)	50.0 g/l
H_3PO_4 (85%)	21 ml/l
$CaSO_4.2H_2O$	0.9 g/l
K_2SO_4	14.28 g/l
$MgSO_4.7H_2O$	11.7 g/l
KOH	3.9 g/l
Peptone	10.0 g/l
[1]Yeast Extracts	5.0 g/l
[2]Minerals and Trace Metals	1.0 ml/l

[1]Yeast extract is Amberex TM 1003 which is available from and a trademark of Universal Foods Corporation, Milwaukee, Wisconsin.
[2]Minerals and trace metals are $FeSO_4.7H_2O$ 65.0 g/l, $CuSO_4.5H_2O$ 6.0 g/l, $ZnSO_4.7H_2O$ 20 g/l, $MnSO_4$ 3.0 g/l and H_2SO_4 5.0 ml/l

The yeast extracts utilized in the present invention include but are not limited to yeast extracts selected from the group consisting of Amberex TM 1003 and Bacto TM Yeast Extract (Difco Laboratories Incorporated). Alternatively, corn steep liquor could be used to replace yeast extracts as a source of nitrogen.

Trace metals utilized in the present invention are those trace metals generally utilized in the yeast growth provided in an amount sufficient to not limit the growth rate or HSA production of *Pichia pastoris* which include but are not limited to trace metals selected from the group consisting of cobalt, molybdenum, iron, copper, zinc, and manganese.

The fermentation temperature should generally range from about 20° C. to about 35° C. and preferably should be about 30° C.

The dissolved oxygen content in the fermentation vessel where the fermentation is conducted in a batch-fed manner may range from about 20 percent to about 80 percent of saturation and preferably will range from about 30 percent to about 60 percent of saturation.

After the *Pichia pastoris* strains transformed with a vector containing the HSA structural gene have been cultivated to a high density, the transformed strains should then be induced to express HSA at a pH of from about 5.7 to about 6.0. For example, if this technique is employed with a strain transformed with a linear expression cassette containing a methanol inducible regulatory region, the culture would first be grown to the desired density on minimal salts, biotin and 5 percent glycerol by weight. The pH should be adjusted to 5.8 (with ammonia) with a temperature of about 30° C. and a dissolved oxygen concentration of about 20 percent of saturation. After the glycerol is exhausted, the promoter would be induced by beginning a slow methanol feed. The fed should provide methanol to the culture at a rate at least sufficient to maintain the viability of the culture but the maximum methanol concentration in contact with the culture should be no more than about 5.0 percent by weight. The HSA secretion can be monitored during the methanol feeding by sampling the HSA present in the cell free broth. Suitable test for quantifying the amount of HSA produced are known to those skilled in the art, such as running polyacrylamide gels. The methanol feed should be continued until the HSA concentration reaches an acceptable level. Generally, the HSA production will peak after about 120 hours on methanol feed.

If the transformed *Pichia pastoris* cells are grown in shake tubes or shake flasks instead of pH controlled fermenter, additional steps should be taken to assure the maximum yields of secreted proteins, such as HSA. Specifically, it is recommended that the media used be modified from that used in fermenter to a complex media and the aeration be increased. The complex media utilized in the shake flasks and shake tubes should contain added amino acids. The amino acids may be in a defined media containing glutamic acid, methionine, lysine, leucine, isoluecine and other amino acids or through a complex media supplement, such as yeast extract or casamino acids. The relative concentrations of the added amino acids should generally range from about 2.5 mg/liter to about 10 mg/liter with the preferred range being from about 4 mg/liter to about 6 mg/liter of glutamic acid, methionine, lysine, leucine and isoluecine and from about 0.5 mg/liter to about 3 mg liters of the remaining amino acid (however, histidine may be omitted entirely from the added amino acids). If yeast extract is used in place of the added amino acids, it is preferred that the yeast extract be provided in a concentration of in the range of from about 1 g/liter to about 15 g/liter be utilized in the media and most preferably the yeast extract will be provided in a concentration of 10 g/liter. It has also been found desirable to add peptone to the media to improve secretion in shake tubes and shake flasks. For optimum secretion that peptone be used with the yeast extract in a concentration of from in the range of from about 1 g/liter to about 50 g/liter, and most preferably in a concentration of about 20 g/liter. As a guideline, it is generally recommended that the peptone concentration be twice the yeast extract concentration.

Aeration in shake flask and shake tube growth of transformed *Pichia pastoris* appears to be an important parameter in obtaining optimum secretion. To insure adequate aeration, it is recommended that shake tube or flask have a large aperture covered with an air permeable cap. Suitable air permeable caps can be made of a loose filter material, such as cheese cloth. One suitable shake flask for this invention is the Tunair shake flask. Generally, low baffle shake flasks are also recommended to avoid excessive foaming. Shaker speed for aeration is recommended to be in the range of from about 250 rpms to about 300 rpms.

After a suitable cell density is achieved in the shake flask or shake tube, the cells may be recovered then resuspended in a medium containing methanol in place of the carbon source used for growth to induce the secretion of protein. The flask or shake tubes may then be monitored on a regular basis to determine when the desired level of production has been achieved.

The invention will now be described in greater detail in the following non-limiting examples.

EXAMPLES

General information pertinent to the Examples:

Stains

Pichia pastoris GS115 (his 4) NRRL Y-15851

E. coli DG75' (hsd1, leu6, lacY, thr-1, supE44, -tonA21, lambda−).

Buffers, Solutions and Media

The buffers, solutions, and media employed in the following examples have the compositions given below:

dH_2O	deionized H_2O that has been treated with a milli-Q (Millipore) reagent water system.
1M Tris buffer	121.1 g Tris base in 800 mL of H_2O; adjust pH to the desired value by adding concentrated (35%) aqueous HCl; allow solution to cool to room temperature before final pH adjustment, dilute to a final volume of 1 L.
TE buffer	1.0 mM EDTA in 0.01 M (pH 8.0) Tris buffer
SED	1 M sorbitol 25 mM EDTA 50 mM DTT, added prior to use –adjust to pH 8
SCE	9.1 g sorbitol 1.47 g Sodium citrate 0.168 g EDTA –pH to 5.8 with HCl in 50 ml dH_2O and autoclave
CaS	1 M sorbitol 10 mM $CaCl_2$ –filter sterilize
SOS:	1 M sorbitol 0.3x YPD 10 mM $CaCl_2$
PEG	20% polyethylene glycol-3350 10 mM $CaCl_2$ 10 mM Tris-HCl (pH 7.4) –filter sterilize
Solution A	0.2 M Tris-HCl (pH 7.5) 0.1 M $MgCl_2$ 0.5 M NaCl 0.01 M dithiothreitol (DTT)
Solution B	0.2 M Tris-HCl (pH 7.5) 0.1 M $MgCl_2$ 0.1 M DTT
Solution C (keep on ice)	4 µl solution B 4 µl 10 mM dATP 4 µl 10 mM dTTP 4 µl 10 mM dGTP 4 µl 10 mM dCTP 4 µl 10 mM ATP 5 µl T_4 ligase (2 U/µl) 12 µl H_2O Recipe for Solution C was modified from Zoller & Smith
LB Broth, 1 liter	5.0 g yeast extract 10.0 g tryptone

-continued

	5.0 g NaCl
10X Transfer Buffer	96.8 g Trizma Base
	9.74 g glycine
	water to 1 liter
Ligation Buffer	50 mM Tris-HCl (pH 7.4)
	10 mM $MgCl_2$
	10 mM dithiothreitol
	1 mM ATP
Phosphatase Buffer	50 mM Tris-HCl (pH 9.0)
	1 mM $MgCl_2$
	1 mM $ZnCl_2$
	1 mM spermidine
Bsu36I buffer	100 mM NaCl
	10 mM Tris-HCl (pH 7.4)
	10 mM $MgCl_2$
	100 μg/ml BSA
Csp45I buffer	60 mM NaCl
	10 mM Tris-HCl, pH 7.5
	7 mM $MgCl_2$
	100 μg/ml BSA
REact 1 buffer	50 mM Tris-HCl, pH 8.0
	10 mM $MgCl_2$
	100 μg/ml BSA
REact 2 buffer	REact 1 buffer + 50 mM NaCl
REact 3 buffer	REact 1 buffer + 100 mM NaCl
HS buffer	50 mM Tris-HCl, pH 7.5
	10 mM $MgCl_2$
	100 mM NaCl
	1 mM DTT
	100 μg/ml BSA
10X Basal Salts	42 mls Phosphoric Acid, 85%
	1.8 g Calcium Sulfate.$2H_2O$
	28.6 g Potassium Sulfate
	23.4 g Magnesium Sulfate.$7H_2O$
	6.5 g Potassium Hydroxide
Ptm_1 Trace Salts Solution	6.0 g Cupric Sulfate.$5H_2O$
	0.08 g Sodium Iodide
	3.0 g Manganese Sulfate.H_2O
	0.2 g Sodium Molybdate.H_2O
	0.02 g Boric Acid
	0.5 g Cobalt Chloride
	20.0 g Zinc Chloride
	65.0 g Ferrous Sulfate.H_2O
	0.20 g Biotin
	5.0 mls Sulfuric Acid
YPD (yeast extract peptone dextrose medium)	10 g bacto yeast extract
	20 g peptone
	10 g dextrose
	water to 1 liter
MGY (minimal glycerol medium)	13.4 g yeast nitrogen base with ammonium sulfate, and without amino acids
	400 μg biotin
	10 ml glycerol
	water to 1 liter
MM (minimal methanol medium)	Same as MGY, except that 5 ml methanol is used in the place of 10 ml glycerol.
SDR (supplemented dextrose regeneration medium):	13.4 g yeast nitrogen base with ammonium sulfate and without amino acids
	400 μg biotin
	182 g sorbitol
	10 g glucose
	2 g Histidine assay mix (Gibco)
	50 mg glutamine
	50 mg methionine
	50 mg lysine
	50 mg leucine
	50 mg isoleucine
	10 g agarose
	water to 1 liter
BMGR (Buffered minimal glycerol-enriched medium)	100 ml/liter Potassium phosphate buffer, (pH 6.0)
	13.4 grams/liter Yeast nitrogen base with ammonium sulfate
	400 μg/liter biotin
	10 ml/liter glycerol
	Amino acids
	glutamic acid, methionine, lysine, leucine and isoleucine: each at 5 mg/liter;
	all the other amino acids except histidine at 1 mg/liter
	Nucleotides
	adenine sulfate, guanine hydrochloride, uracil, and xanthine, each at 40 μg/liter
	Vitamins
	thiamine hydrochloride, riboflavin, and calcium pantothenate, each at 2 μg/liter;
	pyridoxide hydrochloride and nicotinic acid, each at 4 μg/liter;
	pyridoxamine hydrochloride and pyridoxal hydrochloride, each at 1 μg/liter;
	para-amino benzoic acid at 0.3 μg/liter;
	folic acid at 0.03 μg/liter
	Trace minerals
	magnesium sulfate at 800 μg/liter;
	ferrous sulfate at 40 μg/liter;
	manganese sulfate at 80 μg/liter;
	sodium chloride at 40 μg/liter
BMGY (Buffered minimal glycerol-complex medium)	100 ml/liter potassium phosphate buffer, (pH 6.0)
	13.4 grams/liter yeast nitrogen base with ammonium sulfate and without amino acids
	biotin at 400 μg/liter
	glycerol at 10 ml/liter
	yeast extract at 10 g/liter
	peptone at 20 g/liter
BMMR (Buffered minimal methanol-enriched medium)	Same as BMGR, with the exception that 5 ml methanol/liter is added in the place of glycerol
BMMY (Buffered minimal methanol-complex medium)	Same as BMGY, with the exception that 5 ml methanol/liter is added in the place of glycerol

Techniques

Suitable techniques for recombinant DNA lab work may be found in many different references including but not limited to: *Methods in Enzymology*, (Orlando, Fla.: Academic Press, Inc.), particularly Volume 152, published as, *Guide to Molecular Cloning Techniques*, by Berger and Kimmel (Orlando, Fla.: Academic Press, Inc., 1987) and *Molecular Cloning/A Laboratory Manual*, by Sambrook et al., 2d ed. (Cold Spring Harbor Laboratory Press, 1989) and which are all hereby incorporated by reference.

EXAMPLE I

Construction of 5'-exact HSA expression vector pHSA313

The pHSA313 vector was constructed to provide a vector with an exact linkage between the 3' end of the native AOX1 5' regulatory region (promoter) and the start codon of the HSA structural gene.

A. Creation of pHSA113ΔCla

About 200 ng of pHSA113, disclosed in European Patent Application 0 344 459 which is herein incorporated by reference, (see FIG. 7) was digested at 37° C. for 1 hour with 1 unit of ClaI in 20 μl of REact 1 buffer. The digestion mixture was brought to 100 μl with water and extracted once with an equal volume of phenol:-chloroform:isoamyl alcohol (25:24:1 V/V), followed by extracting the aqueous layer with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA in the aqueous phase was precipitated by adjusting the NaCl concentration to 0.2M and adding 3 volumes of cold ethanol. The mixture was allowed to stand on ice (4° C.) for 10 minutes and the DNA precipitate was collected by centrifugation for 30 minutes at 10,000×g in a microfuge at 4° C. The DNA pellet was washed 2 times with 70% aqueous cold ethanol. The washed pellet was vacuum dried and dissolved in 10 μl water to which 2 μl of 10×ligation buffer, 2 μl of 1 mg/ml BSA, 6 μl of water and 1 unit T_4 DNA ligase were added. The mixture was incubated overnight at 4° C. and a 10 μl aliquot

was used to transform *E. coli* DG75' (Maniatis, et al.) to obtain pHSA113ΔCla, which represents the deletion of HIS4 and 3'AOX1, along with small stretches of pBR322 sequences used to link these sequences. The deletion of the HIS4, 3' AOX1 and pBR322 sequences removes one of two Csp45I sites present in the pHSA113 vector. The remaining Csp45I site is in the AOX1 5' regulatory region (promoter).

B. Creation of pXHSA113ΔCla

Digest 5 μg of pHSA113ΔCla for 1 hour at 37° C. with 10 units of BstEII in 100 μl of REact 2 buffer. The digestion mixture was extracted with phenol and precipitated as detailed in step A. The DNA precipiate was dissolved in 100 μl of Csp45I buffer and digested at 37° C. for 2 hours in the presence of 10 units of Csp45I. The digested DNA was then phenol extracted and precipitated as described in step A. The DNA precipitate was dissolved in 20 μl of water and 10 μl aliquots were loaded on 2 neighboring wells of a 0.9% agarose gel. Following electrophoresis, the gel portion corresponding to one of the lanes was stained and this was used to locate the position of the Csp45I-BstEII fragment of pHSA113ΔCla in the unstained lane. The gel portion containing the larger Csp45I-BstEII fragment was excised out and the DNA in the gel was electroeluted into 500 μl of 5 mM EDTA, pH 8.0. The DNA solution was phenol extracted as detailed in step A and the DNA precipitate was dissolved in 100 μl water. The larger Csp45I-BstEII fragment was then ligated with the BstEII-Csp45I oligonucleotide linker described below. An aliquot (10 μl) was ligated overnight at 4° C. with 20 ng of annealed linker oligonucleotides 5'-CGAAACG ATG AAG TGG (SEQ ID NO:4) and 5'-GTTACC-CACTTCATCGTTT (SEQ ID NO:5) in 20 μl ligase buffer containing 100 μg/ml BSA and 1 unit of T_4 DNA ligase. The ligation mixture was used to transform *E. coli* DG75' to obtain pXHSA113ΔCla. The pXHSA113ΔCla vector by virtue of the linker described above has an exact linkage between the 3' end of the native AOX1 5' regulatory region (promoter) and the HSA ATG start codon with no extraneous DNA sequences.

C. Creation of pHSA313

1 μg of pXHSA113ΔCla was digested for 4 hours at 37° C. with ClaI in 100 μl of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200 μl reaction volume for 30 minutes at 37° C. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in water at a concentration of approximately 10 ng/μl as described in step A and stored at −20° C.

1 μg of pA0807N (FIG. 8, construction of which is described in European Patent Application 0 344 459) was digested for 4 hours at 37° C. with PstI in 100 μl of REact 2 buffer. The digested DNA adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200 μl reaction volume for 15 minutes at 55° C. At the end of the 15 minutes another 10 units of phosphatase was added and incubated for 15 minutes. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated as described in step A. DNA was digested for 4 hours at 37° C. with 5 units of ClaI in 100 μl buffer containing 100 μg/ml BSA, followed by phenol extraction and precipitation of DNA as outlined in step A. The DNA precipitate was dissolved in water at a concentration of approximately 20 ng/μl.

Approximately 100 ng (10 μl) of ClaI cleaved-phosphatased pXHSA113ΔCla was mixed with approximately 80 ng of PstI digested-phosphatased and ClaI-cleaved pA0807N (4 μl), 4 μl of 5× ligase buffer, 2 μl of 1 mg/ml BSA and ligated overnight at 4° C. using 1 unit of T_4 DNA ligase. The ligation mixture was used to transform *E. coli* DG75' to obtain pHSA313. The pHSA313 plasmid from this ligation contains the complete pXHSA113ΔCla sequence linked to the HIS4 gene and the AOX1 3' second insertable sequence derived from A0807N. The relative orientation of the components of the pHSA313 plasmid is the same as that shown in FIG. 7 for plasmid pHSA113.

EXAMPLE II

Construction of Expression Vector pPGP1

The expression vector pPG1 was constructed in the following manner. pXHSA113ΔCla (see Example I) was digested with Bsu36I and PvuII (partial) and the vector backbone was isolated. An HSA structural gene on a PvuII-Bsu36I fragment analogous to the structural gene contained in pHSA113 (disclosed in European Patent Application 0 344 459) was ligated to this vector backbone to obtain pPGP1ΔCla. About 100 ng of pPGP1ΔCla was digested with ClaI at 37° C. for 1 hour. The DNA was recovered as in Example I. About 100 ng of pA0807N (shown herein in FIG. 8 and disclosed in European Patent Application 0 344 459) was digested with PstI, alkaline phosphatase treated and then digested with ClaI as detailed in Example I C. This fragment was then ligated to ClaI cleaned, alkaline phosphatase treated pPGP1ΔCla to obtain pPGPI. (GS115 pPGP1-9-6 is a clone which was obtained by transformation of *Pichia pastoris* GS115 with pPGP1 and this clone was used in fermentation).

EXAMPLE III

Construction of 5' and 3' exact HSA expression plasmid pHSA413

The pHSA413 vector was constructed to provide a vector with an exact linkage between the 3' end of the AOX1 5' regulatory region and the start codon fo the HSA structural gene as well as an exact linkage between the 5' end of the AOX1 3' termination sequence and the 3' end of the HSA structural gene.

A. Creation of pXXHSA113ΔCla

1 μg of pXHSA113ΔCla was digested for 4 hours at 37° C. with 10 units of EcoRI in 100 μl REact 3 buffer. The digestion mixture was phenol extracted and DNA precipitated as detailed in Example VI. DNA precipitate was dissolved in 20 μl water and digested for 1 hours at 37° C. with 20 units of Bsu36I in 100 μl of Bsu36I buffer. The digestion mixture was phenol extracted, DNA precipitated and dissolved in 100 μl of water as detailed in Example VI. Approximately 100 ng of EcoRI and Bsu36I-cleaved DNA was mixed with 10 ng of annealed oligonucleotides 5'-TTAGGCT-TATAAG (SEQ ID NO:6) and 5'-AATTCT-TATAAGCC (SEQ ID NO:7) and ligated overnight at 4° C. in 20 μl of T_4 DNA ligase buffer containing 100 μg/ml BSA and 10 units of T_4 DNA ligase. The ligation mixture was used to transform *E. coli* to obtain pXXHSA113ΔCla. In this plasmid the sequence between Bsu36I and EcoRI (SEQ ID NO:8) present in pxHSA113ΔCla shown below

Bsu36I

5'CCTTAGGCTTATAACATCTCTACATTTAAAAGCATCTCAGCCTACCATG

AGAATAAGAGAAAGAAAATGAAGATCA

AAAGCTTATTCATCTGTGTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCT

GTCTAAAAAACATAAATTTCTTTAATC

ATTTTGCCTCTTTTTCTCTGTGCTTCAATTAATAAAAAATGGAAAGAATCT

AAAAAAAAAAAAAAAAAAAGGAATTC

EcoRI

is replaced by 5'CC TTA GGC TTA TAA GAATTC (SEQ ID NO: 9)

Bsu36I EcoRI

B. Creation of pHSA413

1 μg of pXXHSA113ΔCla was digested for 4 hours at 37° C. with ClaI in 100 μl of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in 200 μl reaction volume for 30 minutes at 37° C. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in water at a concentration of approximately 10 ng/μl as described in step A and stored at −20° C.

Approximately 100 ng (10 μl) of ClaI cleaved-phosphatased pXXHSA113ΔCla was mixed with approximately 80 ng (4 μl) of PstI digested phosphatased and ClaI-cleaved pA0807N (see paragraph 2 in step 3 of Example VI), 4 μl of 5×ligase buffer, 2 μl of 1 mg/ml BSA and ligated overnight at 4° C. using 1 unit of T_4 DNA ligase. The ligation mixture was used to transform *E. coli* DG75' to obtain pHSA413. The pHSA413 plasmid from this ligation contains the complete pXHSA113ΔCla sequence linked to the HIS4 gene and the AOX1 3' second insertable sequence derived from A0807N. The relative orientation of the components of the pHSA413 plasmid is the same as that shown in FIG. 7 for plasmid pHSA113.

EXAMPLE IV

Transformation of *Pichia pastoris* with pHSA313, pHSA413, and pPGP1

A. Vector preparation

About 10 μg each of pHSA313, pHSA413, pPGP1, and pA0807N (negative control) were digested for 12 hours at 37° C. in 200 μl of HS buffer with 50 units of NotI. The digested DNA samples were phenol extracted, precipitated as described in Example VI, dissolved in 20 μl of CaS, and were then used for transformation of *Pichia pastoris* GS115. About 10 μg each of pHSA313, pHSA413, and pA0807N were also digested with 20 units of SstI for 12 hours at 37° C. in 200 μl of REact 2 buffer containing 100 μg/ml of BSA. The digested DNA samples were extracted with phenol, precipitated as described in Example VI and dissolved in 20 μl of CaS.

B. Cell Growth

Pichia pastoris GS115 (NRRL Y-15851) was inoculated into about 10 ml of YPD medium and shake cultured at 30° C. for 12-20 hours. 100 ml of YPD medium was inoculated with a seed culture to give an OD_{600} of about 0.001. The medium was cultured in a shake flask at 30° C. for about 12-20 hours. The culture was harvested when the OD_{600} was about 0.2–0.3 by centrifugation at 1555 g for 5 minutes using a Sorvall RB5C.

C. Preparation of Spheroplasts

The cells were washed in 10 ml of sterile water, and then centrifuged at 1500 g for 5 minutes. (Centrifugation is performed after each cell wash at 1500 g for 5 minutes using a Sorvall RT6000B unless otherwise indicated.) The cells were washed once in 10 ml of freshly prepared SED, once in 10 ml of sterile 1M sorbitol, and finally resuspended in 10 ml of SCE buffer. 7.5 μl of 3 mg/ml Zymolyase (100,000 units/g, obtained from Miles Laboratories) was added to the cell suspension. The cells were incubated at 30° C. for about 10 minutes. (A reduction of 60% in OD_{600} in 5% SDS can be utilized as a correct time marker.) The spheroplasts were washed in 10 ml of sterile 1M sorbitol by centrifugation at 700 g for 5–10 minutes. 10 ml of sterile CaS was used as a final cell wash, and the cells were centrifuged again at 700 g for 5–10 minutes and then resuspended in 0.6 ml of CaS.

D. Transformation

Pichia pastoris GS115 cells were transformed with 10 μg of linearized DNA (see step A) using the spheroplast transformation technique of Sreekrishna et al, *Gene* 59, 115-125 (1987). DNA samples were added (up to 20 μl volume) to 12×75 mm sterile polypropylene tubes. (DNA should be in a suitable buffer such as TE buffer or CaS.) 100 μl of spheroplasts were added to each DNA sample and incubated at room temperature for about 20 minutes. 1 ml of PEG solution was added to each sample and incubated at room temperature for about 15 minutes and centrifuged at 700 g for 5–10 minutes. SOS (150 μl) was added to the pellet and incubated for 30 minutes at room temperature. Finally 850 μl of 1M sorbitol was added.

E. Regeneration of Spheroplasts

A bottom agarose layer of 20 ml of regeneration agar SDR was poured per plate at least 30 minutes before transformation samples were ready. In addition, 8 ml aliquots of regeneration agar were distributed to 15 ml conical bottom Corning tubes in a 45° C. water bath during the period that transformation samples were in SOS. Aliquots of 50 or 250 μl of the transformed sample was added to the 8 ml aliquots of molten regeneration agar held at 45° C. and poured onto plates containing the solid 20 ml bottom agar layer. The plates were incubated at 30° C. for 3–5 days.

F. Selection of Transformants

Transformants were selected for by culturing on SDR, a media lacking histidine. The colonies which grew in the absence of histidine were also screened for

"methanol-slow" phenotype, indicating displacement of the AOX1 structural gene by the NotI DNA fragment) in the case of transformants obtained using NotI linearized vectors. Several transformed GS115 cells showing "methanol-normal" (those obtained with SstI linearized DNA) and methanol-slow were then cultured and assayed for the production of HSA.

EXAMPLE V

Methanol Induced Secretion of HSA in *Pichia pastoris* Integrative Transformants

Pichia pastoris GS115 strains transformed with pHSA313, pHSA413, and pPGP1 were analysed for HSA secretion in shake tube cultures. Both methanol-slow and methanol-normal strains were used. In each case 36 independent clones were studied. Transformants obtained with pA0807N served as negative controls. A protocol was developed to ensure efficient secretion and stable accumulation of HSA in the culture medium.

Cells were grown to saturation in 10 ml BMGR or BMGY, and were placed in 50 ml tubes (2–3 days). The cells would be in the range of 10–20 A_{600} units. The cells were harvested, the supernatant liquid was discarded, and then the pellet was resuspended in 2 ml of BMMR or BMMY. The tube was covered with a sterile gauze (cheese cloth) instead of a cap. The tube(s) were then returned to a 30° C. shaker. At the end of 2–3 days, the cells were pelleted, and the supernatant assayed for product. The pellets could be resuspended with fresh medium and returned to the shaker for renewed secretion. With Pichia-HSA strains, 10 µl of media supernatant was sufficient for analysis by SDS-PAGE followed by Coomassie staining. Under these conditions a single band of 67 kD corresponding to HSA was observed. There was no significant difference between the expression levels of GS115/pHSA313 vs GS115/pHSA413 transformants, suggesting that deleting the 3' untranslated sequences from the HSA gene present in pHSA313 did not significantly affect expression levels. No significant difference in the HSA expression level was observed between methanol-slow vs methanol-normal transformants, suggesting that disruption of AOX1 was not essential for efficient HSA expression. As expected, HSA was absent in both the culture medium and the cell extract of GS115/pAO807N transformants (negative control). Clonal variants were selected which demonstrated increased levels of HSA secretion.

EXAMPLE VI

Batch-Fed Fermentation of Mut *Pichia pastoris* for Production of HSA

Pichia pastoris GS115:pHSA 413-6 and pPGP1-9-6 were inoculated into two 20 liter Biolafitte fermenters with an 8.5 l working volume. The inoculum was prepared in the following manner: a culture was grown on a YM plate and then transferred to 100 ml YM broth in a shake flask and grown for about 24 hours. 50 mls of this culture was transferred to 1 liter of YM broth in a shake flask and also grown for about 24 hours. 1 liter of this was then transferred to 8.5 liters of fermenter medium in the Biolafitte fermenter. Fermentor medium consisted of Minimal salts + biotin + 5 percent glycerol. Batch growth conditions included the following: pH = 5.8 (controlled with NH_3), temperature = 30° C., and percent dissolved oxygen greater than 20 percent air saturation.

Glycerol exhaustion was complete after about 24 hours, at which time a slow methanol feed was begun at a rate of 10–15 ml/hr. The methanol concentration was monitored in the fermenter and the feed rate was adjusted to maintain a concentration of 0.5–0.9 percent of methanol in the broth.

Secreted HSA in the media was measured quantitatively by densitometry of Coomassie blue stained polyacrylamide gels containing SDS (SDS-PAGE). Areas were referenced to a series of known weights of authentic HSA run on the same SDS-PAGE gels. The data from these gels is included in Tables I and II.

The following Table illustrates the effect of changes in pH on the amount of HSA produced:

TABLE III

Production of HSA by Batch-Fed Fermentation

Run	Strain	pH	HSA g/l
1	GS115:pPGP1-9-6	5.09–5.32	0.71
2	GS115:pPGP1-9-6	5.22	0.81
3	GS115:pPGP1-9-6	5.91	1.28
4	GS115:pPGP1-9-6	5.78	1.59
5	GS115:pPGP1-9-6	5.78	1.98
6	GS115:pPGP1-9-6	5.79	1.32

The following Table illustrates the level of HSA production which can be achieved at higher pH levels:

TABLE IV

Production of HSA by Batch-Fed Fermentation

Run	Strain	pH	Hours MeOH	Dry Cell Wt.	HSA Broth g/l
1	GS115:pHSA 413-6	5.79	101	ND	2.13
2	GS115:pHSA 413-6	5.85	237	101	3.39
3	GS115:pHSA 413-6	5.85	265	98	2.70
4	GS115:pHSA 413-6	5.97	258	117	2.90

ND = Not Determined

EXAMPLE VII

Protocol for Shake Tube and Shake Flask Secretion of Proteins from *P. pastoris*

For efficient secretion and stable accumulation of HSA in shake tubes and shake flasks it is necessary to use a pH of 5.7–6.4 instead of 5.0 or 5.2 for the fermenter media, to add small amounts yeast extract (0.5–0.1%) and peptone (0.1–0.2%) to the fermenter medium and to start inducing expression at a low cell density (20–25 gram dry cell weight/liter). Using these techniques, we have developed a protocol that permits efficient secretion of HSA from cells grown in shake tubes and flasks. We believe that this protocol is applicable in general to secretion of proteins from *Pichia pastoris*.

Shake Tube

Grow cells to saturation in 10 ml BMGR or BMGY placed in 50 ml tube (2–3 days). The A_{600} of cells will be in the range of 10–20. Harvest cells, discard the supernatant liquid and resuspend the pellet with 2 ml of BMMR or BMMY. Cover the tube with a sterile gauze or cheese cloth instead of the cap, Return the tube(s) to the shaker and maintain the shaker at about 30° C. At the end of 2–3 days, pellet cells, and analyze supernatant for product. The pellet can be resuspended with fresh media and returned to shaker for renewed secretion. With Pichia-HSA strains, 10 ul of media supernatant is

sufficient for analysis by SDS-PAGE followed by Coomassie staining. Under these conditions, a single band corresponding to HSA size (67 kD) is observed.

Shake Flask

Grow cells as described above in 1 liter of medium (BMGY or BMGR) in a 2 liters flask. Harvest cells and suspend with 50–75 ml of BMMR or BMMY in a fermenter flask (Tunair TM shake-flask fermentation system, Research Products International Corporation) or a baffled flask covered with cheese cloth. Return to the shaker at 30° C. and induce for 2–4 days. At the end of 2–4 days the cells are pelleted and the supernatant is analyzed for product. Shake tubes secretion can be re-initiated by resuspending the pelleted cells in fresh media.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 9

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 940 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Genomic DNA

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGATCTAACA TCCAAAGACG AAAGGTTGAA TGAAACCTTT TTGCCATCCG ACATCCACAG   60
GTCCATTCTC ACACATAAGT GCCAAACGCA ACAGGAGGGG ATACACTAGC AGCAGACCGT  120
TGCAAACGCA GGACCTCCAC TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC  180
AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA TTCCTTCTAT TAGGCTACTA  240
ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG GCGAGGTTCA TGTTTGTTTA  300
TTTCCGAATG CAACAAGCTC CGCATTACAC CCGAACATCA CTCCAGATGA GGGCTTTCTG  360
AGTGTGGGGT CAAATAGTTT CATGTTCCCC AAATGGCCCA AAACTGACAG TTTAAACGCT  420
GTCTTGGAAC CTAATATGAC AAAAGCGTGA TCTCATCCAA GATGAACTAA GTTTGGTTCG  480
TTGAAATGCT AACGGCCAGT TGGTCAAAAA GAAACTTCCA AAAGTCGGCA TACCGTTTGT  540
CTTGTTTGGT ATTGATTGAC GAATGCTCAA AAATAATCTC ATTAATGCTT AGCGCAGTCT  600
CTCTATCGCT TCTGAACCCC GGTGCACCTG TGCCGAAACG CAAATGGGGA AACACCCGCT  660
TTTTGGATGA TTATGCATTG TCTCCACATT GTATGCTTCC AAGATTCTGG TGGGAATACT  720
GCTGATAGCC TAACGTTCAT GATCAAAATT TAACTGTTCT AACCCCTACT TGACAGCAAT  780
ATATAAACAG AAGGAAGCTG CCCTGTCTTA AACCTTTTTT TTTATCATCA TTATTAGCTT  840
ACTTTCATAA TTGCGACTGG TTCCAATTGA CAAGCTTTTG ATTTTAACGA CTTTTAACGA  900
CAACTTGAGA AGATCAAAAA ACAACTAATT ATTCGAAACG                         940
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 600 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Genomic DNA

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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AAAGTAAACC CCATTCAATG TTCCGAGATT TAGTATACTT GCCCCTATAA GAAACGAAGG   60
ATTTCAGCTT CCTTACCCCA TGAACAGAAA TCTTCCATTT ACCCCCCACT GGAGAGATCC  120
GCCCAAACGA ACAGATAATA GAAAAAAGAA ATTCGGACAA ATAGAACACT TTCTCAGCCA  180
ATTAAAGTCA TTCCATGCAC TCCCTTTAGC TGCCGTTCCA TCCCTTTGTT GAGCAACACC  240
```

-continued

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ATCGTTAGCC AGTACGAAAG AGGAAACTTA ACCGATACCT TGGAGAAATC TAAGGCGCGA   300
ATGAGTTTAG CCTAGATATC CTTAGTGAAG GGTGTTCCGA TACCTTCTCC ACATTCAGTC   360
ATAGATGGGC AGCTTTGTTA TCATGAAGAG ACGGAAACGG GCATTAAGGG TTAACCGCCA   420
AATTATATAA AAGACAACAT GTCCCCAGTT TAAAGTTTTT CTTTCCTATT CTTGTATCCT   480
GAGTGACCGT TGTGTTTAAT ATAACAAGTT CGTTTTAACT TAAGACCAAA ACCAGTTACA   540
ACAAATTATA ACCCCTCTAA ACACTAAAGT TCACTCTTAT CAAACTATCA AACATCAAAA   600
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1830 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Genomic DNA

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG AAG TGG GTA ACC TTT ATT TCC CTT CTT TTT CTC TTT AGC TCG
Met Lys Trp Val Thr Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser
    -35                 -30                 -25

GCT TAT TCC AGG GGT GTG TTT CGT CGA GAT GCA CAC AAG AGT GAG
Ala Tyr Ser Arg Gly Val Phe Arg Arg Asp Ala His Lys Ser Glu
    -20                 -15                 -10

GTT GCT CAT CGG TTT AAA GAT TTG GGA GAA GAA AAT TTC AAA GCC
Val Ala His Arg Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys Ala
    -5                  1                   5

TTG GTG TTG ATT GCC TTT GCT CAG TAT CTT CAG CAG TGT CCA TTT
Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln Gln Cys Pro Phe
10                  15                  20

GAA GAT CAT GTA AAA TTA GTG AAT GAA GTA ACT GAA TTT GCA AAA
Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu Phe Ala Lys
25                  30                  35

ACA TGT GTT GCT GAT GAG TCA GCT GAA AAT TGT GAC AAA TCA CTT
Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys Ser Lue
40                  45                  50

CAT ACC CTT TTT GGA GAC AAA TTA TGC ACA GTT GCA ACT CTT CGT
His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu Arg
55                  60                  65

GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA AAA CAA GAA CCT
Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
70                  75                  80

GAG AGA AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA AAC
Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn
85                  90                  95

CTC CCC CGA TTG GTG AGA CCA GAG GTT GAT GTG ATG TGC ACT GCT
Leu Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala
100                 105                 110

TTT CAT GAC AAT GAA GAG ACA TTT TTG AAA AAA TAC TTA TAT GAA
Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu
115                 120                 125

ATT GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG GAA CTC CTT TTC
Ile Ala Arg Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe
130                 135                 140

TTT GCT AAA AGG TAT AAA GCT GCT TTT ACA GAA TGT TGC CAA GCT
Phe Ala Lys Arg Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala
145                 150                 155

GCT GAT AAA GCT GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT CGG
Ala Asp Lys Ala Ala Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg
160                 165                 170
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-continued

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GAT GAA GGG AAG GTT TCG TCT GCC AAA CAG AGA CTC AAG TGT GCC
Asp Glu Gly Lys Val Ser Ser Ala Lys Gln Arg Leu Lys Cys Ala
175                 180                 185

AGT CTC CAA AAA TTT GGA GAA AGA GCT TTC AAA GCA TGG GCA GTA
Ser Leu Gln Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val
190                 195                 200

GCT CGC CTG AGC CAG AGA TTT CCC AAA GCT GAG TTT GCA GAA GTT
Ala Arg Leu Ser Gln Arg Phe Pro Lys Ala Glu Phe Ala Glu Val
205                 210                 215

TCC AAG TTA GTG ACA GAT CTT ACC AAA GTC CAC ACG GAA TGC TGC
Ser Lys Leu Val Thr Asp Leu Thr Lys Val His Thr Glu Cys Cys
220                 225                 230

CAT GGA GAT CTG CTT GAA TGT GCT GAT GAC AGG GCG GAC CTT GCC
His Gly Asp Leu Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu Ala
235                 240                 245

AAG TAT ATC TGT GAA AAT CAA GAT TCG ATC TCC AGT AAA CTG AAG
Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu Lys
250                 255                 260

GAA TGC TGT GAA AAA CCT CTG TTG GAA AAA TCC CAC TGC ATT GCC
Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His Cys Ile Ala
265                 270                 275

GAA GTG GAA AAT GAT GAG ATG CCT GCT GAC TTG CCT TCA TTA GCT
Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser Leu Ala
280                 285                 290

GCT GAT TTT GTT GAA AGT AAG GAT GTT TGC AAA AAC TAT GCT GAG
Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala Glu
295                 300                 305

GCA AAG GAT GTC TTC TTG GGC ATG TTT TTG TAT GAA TAT GCA AGA
Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg
310                 315                 320

AGG CAT CCT GAT TAC TCT GTC GTG CTG CTG CTG AGA CTT GCC AAG
Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys
325                 330                 335

ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT GCC GCT GCA GAT CCT
Thr Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro
340                 345                 350

CAT GAA TGC TAT GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT GTG
His Glu Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val
355                 360                 365

GAA GAG CCT CAG AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG
Glu Glu Pro Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu
370                 375                 380

CAG CTT GGA GAG TAC AAA TTC CAG AAT GCG CTA TTA GTT CGT TAC
Gln Leu Gly Glu Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr
385                 390                 395

ACC AAG AAA GTA CCC CAA GTG TCA ACT CCA ACT CTT GTA GAG GTC
Thr Lys Lys Val Pro Gln Val Ser Thr Pro Thr Leu Val Glu Val
400                 405                 410

TCA AGA AAC CTA GGA AAA GTG GGC AGC AAA TGT TGT AAA CAT CCT
Ser Arg Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His Pro
415                 420                 425

GAA GCA AAA AGA ATG CCC TGT GCA GAA GAC TAT CTA TCC GTG GTC
Glu Ala Lys Arg Met Pro Cys Ala Glu Asp Tyr Leu Ser Val Val
430                 435                 440

CTG AAC CAG TTA TGT GTG TTG CAT GAG AAA ACG CCA GTA AGT GAC
Leu Asn Gln Leu Cys Val Leu His Glu Lys Thr Pro Val Ser Asp
445                 450                 455

AGA GTC ACC AAA TGC TGC ACA GAA TCC TTG GTG AAC AGG CGA CCA
Arg Val Thr Lys Cys Cys Thr Glu Ser Leu Val Asn Arg Arg Pro
460                 465                 470

TGC TTT TCA GCT CTG GAA GTC GAT GAA ACA TAC GTT CCC AAA GAG
Cys Phe Ser Ala Leu Glu Val Asp Glu Thr Tyr Val Pro Lys Glu
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-continued

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475                 480                 485

TTT AAT GCT GAA ACA TTC ACC TTC CAT GCA GAT ATA TGC ACA CTT
Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile Cys Thr Leu
490                 495                 500

TCT GAG AAG GAG AGA CAA ATC AAG AAA CAA ACT GCA CTT GTT GAG
Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala Leu Val Glu
505                 510                 515

CTT GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT
Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu Lys Ala
520                 525                 530

GTT ATG GAT GAT TTC GCA GCT TTT GTA GAG AAG TGC TGC AAG GCT
Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys Ala
535                 540                 545

GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG GGT AAA AAA CTT GTT
Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
550                 555                 560

GCT GCA AGT CAA GCT GCC TTA GGC TTA TAA
Ala Ala Ser Gln Ala Ala Leu Gly Leu  .
565                 570
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Oligonucleotide

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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CGAAACG ATG AAG TGG  16
        Met Lys Trp
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Oligonucleotide

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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GTTACCCACT TCATCGTTT  19
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Oligonucleotide

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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TTAGGCTTAT AAG  13
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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: Oligonucleotide

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AATTCTTATA AGCC 14

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Linker Oligonucleotide

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTTAGGCTT ATAACATCTC TACATTTAAA AGCATCTCAG CCTACCATGA GAATAAGAGA 60

AAGAAAATGA AGATCAAAAG CTTATTCATC TGTGTTTTCT TTTTCGTTGG TGTAAAGCCA 120

ACACCCTGTC TAAAAAACAT AAATTTCTTT AATCATTTTG CCTCTTTTTC TCTGTGCTTC 180

AATTAATAAA AAATGGAAAG AATCTAAAAA AAAAAAAAAA AAAAGGAATT C 231

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Oligonucleotide

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTTAGGCTT ATAAGAATTC 20

That which is claimed is:

1. An improved process for the secretion of a HSA protein in transformed *Pichia pastoris* cells comprising:

(a) cultivating in a fermentation broth said transformed *Pichia pastoris* cells which express a HSA structural gene encoding a native HSA secretion signal sequence and a mature HSA protein, wherein the native HSA signal sequence is operably linked to the sequence encoding the mature HSA protein, under conditions suitable for sustaining the viability of said transformed *Pichia pastoris* cells, under suitable conditions for the expression of said HSA protein by said *Pichia pastoris* cells, and

(b) maintaining the pH of said fermentation broth at a pH in the range of from about 5.7 to about 6.4 contemporaneously with the expression of a HSA protein.

2. The process of claim 1 wherein *Pichia pastoris* is transformed with a vector selected from the group consisting of a circular plasmid and a linear plasmid.

3. The process of claim 2 wherein the vector is a linear integrative site-specific vector.

4. The process of claim 3 wherein said linear integrative site-specific vector contains the following serial arrangement:

(a) a first insertable DNA fragment,

(b) at least one marker gene, and at least one expression cassette containing a HSA structural gene encoding a native HSA signal sequence and a mature HSA protein, operably linked to a *Pichia pastoris* AOX1 5' regulatory region and a 3' termination sequence, and

(c) a second insertable DNA fragment;

wherein the order of the marker gene and cassette of component (b) may be interchanged, and the first and second insertable DNA fragments employed are homologous with separate portions of the *Pichia pastoris* genome wherein the insertable fragments are in the same relative orientation as exist in the *Pichia pastoris* genome.

5. The process of claim 4 wherein the first insertable DNA fragment and the second insertable DNA fragment are obtained from the DNA sequence of a gene from *Pichia pastoris* selected from the group consisting of the AOX1 gene, the p40 gene, the DAS gene, the GAP gene, the PHO1 gene and the HIS4 gene.

6. The process of claim 4 wherein said marker gene is selected from the group consisting of HIS4 isolated from *Pichia pastoris*, ARG4 isolated from *Pichia pastoris*, SUC2 isolated from *Saccharomyces cerevisiae*, G418^R gene of Tn903 and G418^R gene of Tn601.

7. The process of claim 4 wherein said plasmid comprises:

(a) the AOX1 5' regulatory region isolated from *Pichia pastoris* operably linked to

(b) a structural gene for HSA encoding a native signal sequence for HSA and a mature HSA protein, wherein the HSA signal sequence is operably linked to the sequence encoding the mature HSA protein operably linked to

(c) the 3' termination sequence of AOX1 isolated from *Pichia pastoris* operably linked to

(d) at least one marker gene, and

(e) a second DNA fragment which is about a 0.19 kilobase sequence of an autonomous replicating DNA sequence.

8. The process of claim **7** wherein said marker gene is HIS4.

9. The process of claim **1** wherein the transformed *Pichia pastoris* cells are grown in a batch-fed manner during the expression of HSA and the pH of the fermentation broth is maintained during expression of the heterologous protein in the range of from about pH 5.7 to about pH 6.0.

10. The process of claim **9** wherein the fermentation broth contains an effective amount of a suitable minimal salts mixture, growth factors and at least one suitable carbon source selected from the group consisting of methanol, glycerol, sorbitol, glucose, fructose and combinations of two or more thereof to maintain the viability of said transformed *Pichia pastoris* cells.

11. The process of claim **10** wherein after the fermentation broth's carbon source is consumed, the transformed *Pichia pastoris* cells are contacted with methanol wherein the methanol is provided at a rate sufficient to maintain the viability of the *Pichia pastoris* cells in contact therewith and the methanol concentration does not exceed about 5.0 percent by weight.

12. The process of claim **9** wherein the pH during the batch-fed growth of the *Pichia pastoris* cells is pH 5.8.

13. A process according to claim **1** wherein said fermentation broth containing the transformed *Pichia pastoris* cells is contacted with from 2.5 mg/liter to about 10 mg/liter of added amino acids selected from the group consisting of glutamic acid, methionine, lysine, leucine and isoleucine and from about 1 gram/liter to about 50 grams/liter of peptone when said secretion is carried out in a shake tube or shake flask.

14. The process of claim **13** wherein the amino acids are provided in the form of yeast extract at a concentration in the range of from about 1 g/liter to about 15 g/liter.

15. The process of claim **14** wherein the peptone is provided at a concentration of about 20 g/liter.

16. An improved process for the secretion of HSA in transformed cells of *Pichia pastoris* GS115 comprising:

(a) cultivating in a fermentation broth cells of *Pichia pastoris* GS115 which have been transformed with a linear integrative site-specific vector containing the following serial arrangement: a first insertable DNA fragment 5' AOX1 Promoter at least one expression cassette containing a HSA structural gene encoding a signal sequence and a mature HSA protein operably linked to a *Pichia pastoris* AOX1 regulatory region and an AOX1 termination sequence, at least one marker gene and a second insertable DNA fragment 3' to AOX1 termination sequence, under conditions suitable for sustaining the viability of said transformed cells of *Pichia pastoris* GS115, under suitable conditions for the expression of said HSA by said cells of *Pichia pastoris* GS115, and

(b) maintaining the pH of said fermentation broth at a pH in the range of from about 5.7 to about 6.4 contemporaneously with the expression of HSA protein.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,330,901
DATED : July 19, 1994
INVENTOR(S) : William D. Prevatt, et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 5, line 24: "NO." should read --No.--

Column 8, lines 43 & 50: "Amberex TM" should read --Amberex TM--

Column 8, line 51: "Bacto TM" should read --Bacto TM--

Column 13, line 65: before "buffer" insert --REact 1--

Column 14, line 20: "pPG1" should read --pPGP1--

Column 14, line 55: "hours" should read --hour--

Column 17, line 51: "Mut" should read --Mut--

Column 20, line 1: "Tunair TM" should read --Tunair TM--

Column 22, line 26: "Lue" should read --Leu--

Column 28, line 60, Claim 7: "plasmid" should read --vector--

Signed and Sealed this

First Day of August, 1995

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

Goodrick, J.C., *et al.*, "High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system", Biotechnol Bioeng., 74(6):492-497 (2001)

High-Level Expression and Stabilization of Recombinant Human Chitinase Produced in a Continuous Constitutive *Pichia pastoris* Expression System

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Abstract: A continuous fermentation process has been developed in *Pichia pastoris (P. pastoris)* with the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter in order to produce large quantities of recombinant human chitinase (rh-chitinase) for preclinical studies as a potential high-dose antifungal drug. Expression levels of about 200 to 400 mg/L have been demonstrated in fed-batch fermentations using strains with either the traditional methanol-inducible or the constitutive *GAP* promoter. Proteolytic degradation of the enzyme was typically seen in fed-batch fermentations. Continuous production of the enzyme by *P. pastoris* with the *GAP* promoter was demonstrated in a 1.5-L working volume fermentor using either glucose or glycerol as the carbon source. The fermentation could be extended for >1 month with a steady-state protein concentration of approximately 300 mg/L. Cell densities were >400 g/L wet cell weight (WCW) (approximately 100 g/L dry cell weight [DCW]) at a dilution rate (*D*) of 0.83 day^{-1} or 1.2 volume exchanges per day (VVD). No proteolytic degradation of the enzyme was seen in the continuous fermentation mode. *Biotechnol Bioeng* **74**: 492–497, 2001.

Keywords: *P. pastoris* continuous fermentation without methanol induction; stable and high-level expression of recombinant human chitotriosidase or chitinase

INTRODUCTION

Pichia pastoris was recognized in the 1970s as a potential source for production of single-cell proteins for feed supplements due to its rather unique ability to anabolize methanol to very high cell mass. Expression of recombinant proteins in *P. pastoris* has been in development since the late 1980s, and the number of recombinant proteins produced in *P. pastoris* has increased significantly in the past several years (Cregg et al., 1993; Sberna et al., 1996). *P. pastoris* is a desirable expression system because it grows to extremely high cell densities in very simple and defined media free of animal-derived contaminants, and it can secrete expressed proteins at very high levels of >1 g/L and up to 80% of total cellular protein for some proteins (Cregg et al., 1993; Sberna et al., 1996). Unlike bacteria, it is capable of producing complex proteins with posttranslational modifications; for instance, correct folding, glycosylation, and proteolytic maturation (Sberna et al., 1996; White et al., 1994). *P. pastoris* is different than *Saccharomyces cerevisiae* (*S. cerevisiae*) in that it does not tend to hyperglycosylate proteins (oligosaccharide chains of 8–14 mannose) (Grinna and Tschopp, 1989) and the highly immunogenic α1,3-mannose structure is not present (Cregg et al., 1993). *P. pastoris* generally secretes expressed proteins into the medium in a fairly pure form (30% to 80% of total secreted proteins) (Sberna et al., 1996), thus allowing for relatively easy purification. It is also capable of growing in a very wide pH range, from 3 to 7.

Traditionally, *P. pastoris* fermentations are performed in fed-batch mode using the methanol-inducible system (i.e., pPICZα) (Invitrogen, San Diego, CA). Some researchers have adapted this system to continuous or continuous perfusion fermentation with limited success (Digan et al., 1989). Recently, constitutive promoters (*GAP*) have been developed for the *P. pastoris* expression system (Waterham et al., 1997). These vectors allow for continuous production of the desired recombinant protein without methanol induction and are now readily available commercially (Invitrogen). This system is more desirable for large-scale production because the hazard and cost associated with the storage and delivery of large volumes of methanol are eliminated.

One major drawback of the *P. pastoris* system is the degradation of the secreted recombinant protein by its own proteases (Boehm et al., 1999). The situation is worse when high-density fermentation is employed because the concentration of proteases in the fermentation broth may also increase. Several strategies have been tried including the addition of an amino acid–rich supplement, changing of growth pH (pH 3 to 7), and use of a protease-deficient host, but with only limited success.

This article describes the development of a continuous *P.*

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pastoris high-cell-density fermentation system for the production of recombinant human (rh)-chitinase using the *GAP* promoter and the subsequent stabilization of the recombinant protein from proteolytic degradation.

MATERIALS AND METHODS

Cloning and Selection of Human Chitinase (h-chitinase) Gene in *P. pastoris*

Vector Construction

The rh-chitinase cDNA was received from Johannes Aerts, University of Amsterdam (WO 9640940), and used as a template for all PCR reactions. The coding region of h-chitinase, without the secretion signal peptide, and containing *Eco*RI sites at the 5′ and 3′ ends was generated by polymerase chain reaction (PCR) and inserted into *Eco*RI-linearized pPICZα and pGAPZα, which contain the *S. cerevisiae* α-factor secretion signal. The coding region of h-chitinase with its secretion signal peptide and *Eco*RI sites at the 5′ and 3′ ends was generated by PCR and inserted into *Eco*RI-linearized pGAPZα. All vectors were obtained from Invitrogen.

Transformation

P. pastoris cells were made competent and transformed by electroporation as previously described (Becker and Guarente, 1991), with slight modifications. Briefly, *P. pastoris* strains X33 and SMD1168 (Invitrogen) were grown to an OD_{600} of between 0.5 and 0.8 in a 50-mL culture, pelleted, and resuspended in 10 mL of ice-cold 100 m*M* Tris, 10 m*M* ethylene-diamine tetraacetic acid (EDTA) buffer with 200 m*M* dithiothreitol (DTT; Sigma, St. Louis, MO), and then incubated for 15 min at 30°C with shaking at 100 revolutions per minute (rpm). Cells were then washed twice with ice-cold sterile water and once with 1 *M* sorbitol (Invitrogen) and resuspended in 100 μL of 1 *M* sorbitol to a final volume of ≈200 μL. An 80-μL batch of competent cells was electroporated with 2 to 6 of μg DNA in 0.2-cm cuvettes at 1500 V, 25 μF, and 200 Ωusing a BioRad gene pulser with a pulse controller (Bio-Rad Laboratories, Hercules, CA). Immediately after pulsing, 1 mL of ice-cold sorbitol was added to the cuvette. Cells were allowed to recover overnight at room temperature, then plated (20 to 100 μL cells per plate) directly on YPD (yeast extract, peptone, dextrose medium; Invitrogen) agar containing differing amounts of zeocin (Invitrogen) for selection. Plates were incubated at 30°C. Resistant colonies appeared after 2 days on 0.1 to 0.5 mg/mL zeocin and after 3 or 4 days on 1 to 2 mg/mL zeocin.

Selection of High Producers

Several hundred clones that survived higher concentrations (0.5 to 2 mg/mL) of zeocin were screened in test tubes as follows. A single colony was inoculated into 5 mL of YPD in a 50-mL conical centrifuge tube and incubated for 24 h at 30°C with shaking at 250 rpm. Cell density was measured by OD_{600} and a fresh 5 mL of YPD was inoculated with 2.5 × 10^6 cells, then incubated as noted earlier. This process was repeated as necessary until cells from each clone being analyzed were synchronized in growth. Typically, two or three passages was sufficient. Once synchronized, cells were grown for 60 h, as done earlier. Aliquots of culture (50 μL) were aseptically removed at 24, 48, and 60 h and conditioned medium was harvested and analyzed by the *p*-nitrophenol (pNP, Sigma) activity assay, as described in what follows, to identify top producers.

The 1.5-L Fed-Batch Fermentation

A shake flask containing 100 mL of YPD medium was inoculated with one vial (~1 mL, OD_{600} = 25) containing a recombinant *P. pastoris* cell line. The flask was incubated at 30°C with shaking at 220 rpm for 16 to 24 h, until the cell density reached an OD_{600} of >15. YPD medium (pH 6.0) used in shake-flask cultivation consisted of (per liter deionized water): D-glucose (J. T. Baker, Phillipsburg, NJ) 20 g, soy peptone (Quest International, Norwich, NY) 20 g, yeast extract (Quest International) 10 g, yeast nitrogen base (without amino acids) (Difco Laboratories, Detroit, MI) 13.4 g, KH_2PO_4 (J. T. Baker) 11.8 g, K_2HPO_4 (J. T. Baker) 2.3 g, and D-biotin (Sigma) 0.4 mg.

The cells from this flask were used to inoculate a 3.0-L fermentor (Applikon, Foster City, CA) with a 1.5-L working volume at a density of 1.0 to 2.0 OD_{600} units. The fermentor contained basal salts medium plus 2 g/L histidine (Sigma) for His^- strains. Basal salts medium used for fermentor batch cultivation contained (per liter deionized water): glucose (or glycerol) 40 g, H_3PO_4 (85%) 26.7 mL, K_2SO_4 18.2 g, $MgSO_4 \cdot 7H_2O$ 14.9 g, KOH 4.13 g, $CaSO_4 \cdot 2H_2O$ 0.93 g, D-biotin (Sigma) 0.87 mg, trace salts solution 4.35 mL (trace salts solution [per liter deionized water]: $Fe_2(SO_4) \cdot 7H_2O$ 65 g, $ZnSO_4$ 42.19 g, $CuSO_4 \cdot 5H_2O$ 6 g, $MnSO_4 \cdot H_2O$ 3 g, $CoCl_2 \cdot 6H_2O$ 0.5 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, NaI 0.08 g, H_3BO_3 0.02 g) (all components from J. T. Baker, unless noted otherwise). The fermentor was maintained at a temperature of 30°C and agitation rate of 1000 rpm (pH 5.0). The pH was controlled by the addition of phosphoric acid (J. T. Baker) or ammonium hydroxide (J. T. Baker), as needed. Initially, dissolved oxygen (dO_2) levels were maintained at >20% air saturation by the introduction of air to the fermentor at a rate of about 1.0 volume per volume fermentor per minute (VVM). If necessary, 100% oxygen was used to maintain dO_2 at >20% air saturation.

The cells were grown batchwise until the initial glucose was depleted (~24 h) and the WCW was ~80 to 100 g/L. When the initial glucose (or glycerol) was depleted as indicated by a dissolved oxygen (pO_2) spike, fed-batch fermentation was initiated by starting the fed-batch medium at a rate of 0.13 to 0.20 mL/min per liter of initial medium

volume. The fed-batch medium consisted of (per liter deionized water): 500 g D-glucose (or glycerol), 2.4 mg D-biotin, and 12 mL trace salts solution, and, when indicated, 10 g of casamino acids. For the methanol-inducible clones, the glycerol fed-batch phase was continued for about 4 to 6 h, and then a methanol feed was initiated. The methanol feed was ramped up from an initial rate of about 0.065 mL/min per liter to about 0.13 mL/min per liter over a 4-h period. The methanol fed-batch medium consisted of (per liter deionized water): 500 mL of methanol, 2.4 mg of D-biotin, and 12 mL of trace salts solution.

Fed-batch fermentation was continued until activity leveled off (~5 to 7 days). Samples were taken daily for WCW and cell density by OD_{600}. Supernatant was obtained by centrifugation at 4000 to 6000*g* for 25 min at 4°C and stored at −20°C until assayed.

The 1.5-L Continuous Fermentation

After the fed-batch fermentation had been established (see previous subsection), and allowed to continue for approximately 24 h (WCW ~200 to 220 g/L), continuous fermentation was initiated at a D of 0.48 to 0.55 day^{-1} or 0.7 to 0.8 VVD. The continuous feed medium contained (per liter deionized water): 300 g D-glucose, 13.35 mL H_3PO_4 (85%), 9.1 g K_2SO_4, 7.45 g $MgSO_4 \cdot 7H_2O$, 2.07 g KOH, 0.47 g $CaSO_4 \cdot 2H_2O$, 0.87 mg D-biotin, and 4.35 mL trace salts solution. Fermentation conditions were as indicated previously for the fed-batch culture.

After ~24 h of continuous culture, the continuous flow rate was increased to a D of 0.69 to 0.83 day^{-1} or ~1.0 to 1.2 VVD. Flow rate was maintained in this range for the duration of the run. Samples were taken daily for WCW, and cell density by OD_{600}. Supernatant was obtained and stored at −20°C for recombinant protein concentration measurements.

The continuous outflow of culture was harvested daily. Supernatant was obtained by centrifugation at 4000 to 6000*g* for 25 to 35 min at 4°C and stored at −20°C until processing.

rh-Chitinase Activity Assay

Crude supernatant or pNP standard (Sigma) (0 to 20 n*M*/well) was diluted in assay buffer (pH 5.2). One hundred microliters of standard and diluted crude supernatant was placed into duplicate wells in a 96-well microtiter plate. One hundred microliters of substrate, 0.25 mg/mL pNP-β-*N*,*N*′-diacetylchitobiose (Sigma), was then added to each well and the plate incubated at 37°C with shaking at 50 rpm. After 2 h, 50 μL of 1.0N NaOH was added to each well and the absorbance at 405 nm against 650 nm (reference) was read using a microtiter plate reader. Activity was determined using a pNP standard curve. A specific activity (determined using purified material at Genzyme) of 1.67 U/mg was used to convert activity units (units per milliliter) to protein units (milligrams per milliliter).

Sodium Dodecylsulfate–Polymerase Chain Reaction (SDS-PAGE) and Gel Staining

About 10 to 20 μL of supernatant of each sample (~4 μg recombinant protein) was mixed with 20 μL of 2 × SDS-reducing sample loading buffer (BioRad, CA) and 30 to 40 μL was subjected to electrophoresis on 4% to 20% Tris-glycine acrylamide minigels (Ready Gel, Bio-Rad Laboratories) in Tris-glycine–SDS running buffer (Bio-Rad). Gels were stained with Coomassie blue staining reagent (Bio-Rad) for about 1 h, then destained with 40% methanol/10% acetic acid for about 1 h.

RESULTS AND DISCUSSION

Fed-Batch Production of rh-Chitinase by a Methanol-Inducible *Mut*$^+$ Clone (pPICZα-SMD 1168 *His*$^-$)

Cell yield (WCW) of the culture plateaued after 2 days at 200 g/L, whereas activity increased slowly through day 5. Final rh-chitinase concentration in the culture broth reached a moderate level of 300 mg/L (Fig. 1a). However, degrada-

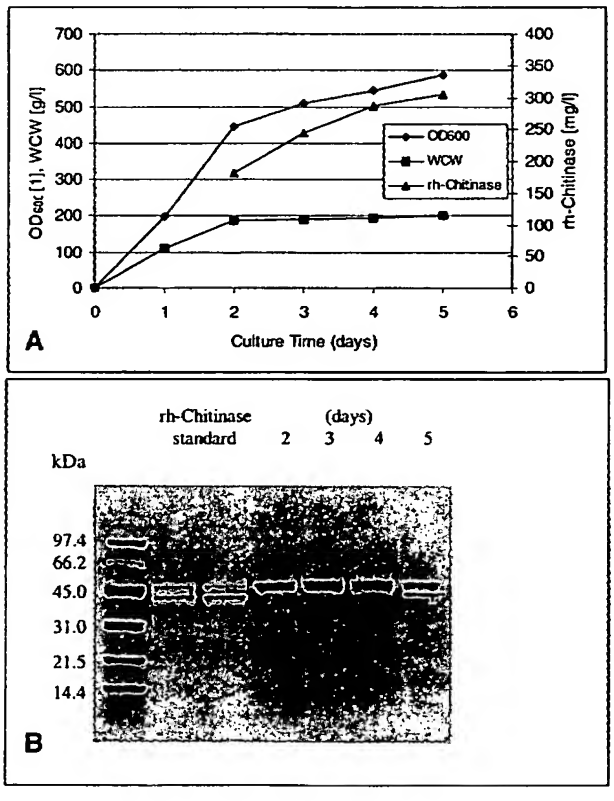

Figure 1. (A) rh-Chitinase expression in methanol induced fed-batch culture with *P. pastoris* (host SMD 1168, *His*$^-$, vector pPICZα). A 50% glycerol solution was fed during day 1 (0.3 mL/min). Subsequently, induction with methanol (0.12 mL/min) was initiated. (B) SDS-PAGE. Lanes 2 and 3: *P. pastoris* derived rh-chitinase-purified standard containing full-length and cleaved 37-kDa protein (both forms are active). Lane 4–7: Supernatant from fed-batch culture, days 2 to 5.

tion of the rh-chitinase was evident on day 4 when the daily samples were analyzed on SDS-PAGE gel (Fig. 1b). Two distinct bands can be seen in samples collected on day 5. These data may explain why the rh-chitinase activity only increased slowly with time under fed-batch mode.

Fed-Batch Production of rh-Chitinase by a Constitutive Clone (pGAPZα-SMD 1168 *His*$^-$)

When pGAPZα-SMD 1168 was grown under fed-batch conditions, cell yield reached 330 g/L WCW and an rh-chitinase concentration of 450 mg/L was attained (Fig. 2a). A degradation pattern similar to the fed-batch methanol-inducible clone (pPICZα-SMD 1168 *His*$^-$) was seen with the recombinant protein. A second lower MW band began to appear after 6 days and the band became more prominent on day 7, suggesting proteolytic degradation (Fig. 2b).

Protection of Enzyme from Proteolytic Degradation by Casamino Acids Supplementation (pGAPZα-SMD 1168 *His*$^-$)

Casamino acids (CAs) have been shown to protect proteins from proteolytic degradation when added to *P. pastoris* cultures (Clare et al., 1991; Werten et al., 1999). They were included in the fed-batch feed medium and supernatant samples (4, 5, 6, and 7 days) were collected and analyzed by SDS-PAGE. A tight band at around 50 kDa in each of the samples analyzed suggests intact rh-Chitinase (Fig. 3). This can be compared with samples from a fed-batch fermentation without CAs, which showed a low-MW band on day 6 (Fig. 2b).

These data suggest that rh-chitinase was most likely degraded by proteolytic enzymes under fed-batch conditions and that rh-chitinase can be stabilized by the addition of CAs. Although CAs appeared to be effective in preventing proteolytic degradation of rh-chitinase in the fermentation broth, this method is not ideal because of the animal origin of CAs.

Stabilization of rh-Chitinase by Continuous Fermentation (pGAPZα-SMD 1168 *His*$^-$)

Figure 4a shows rh-chitinase production and growth data of the constitutive clone (pGAPZα-SMD 1168 *His*$^-$) in a continuous mode. Medium was exchanged at a D of 0.69 day^{-1} or 1.0 VVD. The culture reached steady state on day 2 of continuous mode and rh-chitinase was produced at a volumetric productivity of ~180 mg/L per day. The fermentation was continued for 26 days and samples from days 2 through 8 were analyzed by SDS-PAGE. The gel shows a single rh-chitinase band (~50 kDa) in all samples (Fig. 4b), indicating that continuous fermentation can prevent degradation of rh-chitinase for at least up to 8 days.

It is speculated that, when the culture is grown in continuous mode, the cells do not enter stationary phase and thus little or no proteolytic enzyme(s) is produced and released into the medium. It is also possible that, when the recombinant protein is harvested continuously, it is exposed to less concentrated proteolytic enzymes for a much shorter

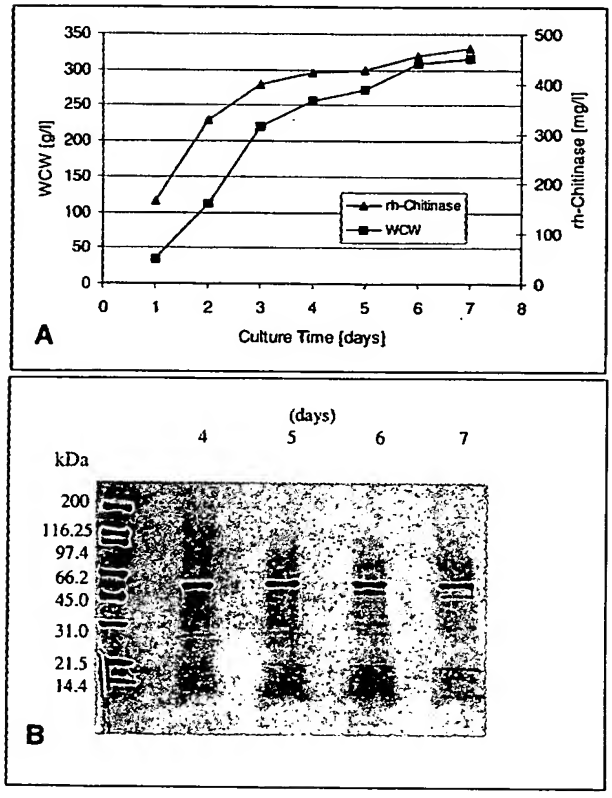

Figure 2. (A) Constitutive rh-chitinase expression in fed-batch culture with *P. pastoris* (host SMD 1168, *His*$^-$, vector pGAPZα). A 50% glycerol solution was fed (0.16 mL/min). (B) SDS-PAGE. Lanes 2–5: supernatant from fed-batch culture, days 4 to 7.

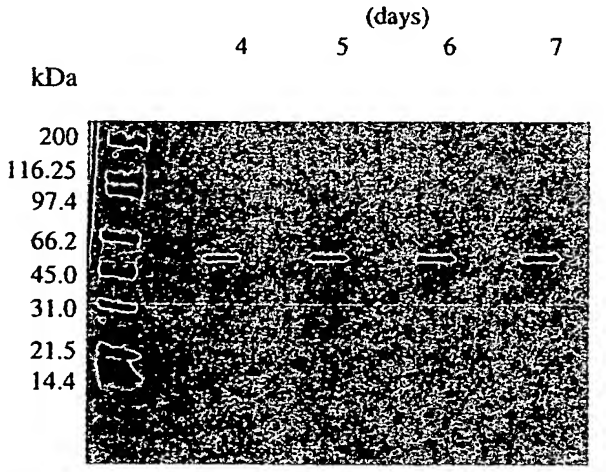

Figure 3. Constitutive rh-chitinase expression in fed-batch culture with *P. pastoris* (host SMD 1168, *His*$^-$, vector pGAPZα). A 50% glycerol solution was fed (0.16 mL/min) containing casamino acids (graph not shown). SDS-PAGE. Lanes 2–5: supernatant from fed-batch culture, days 4 to 7.

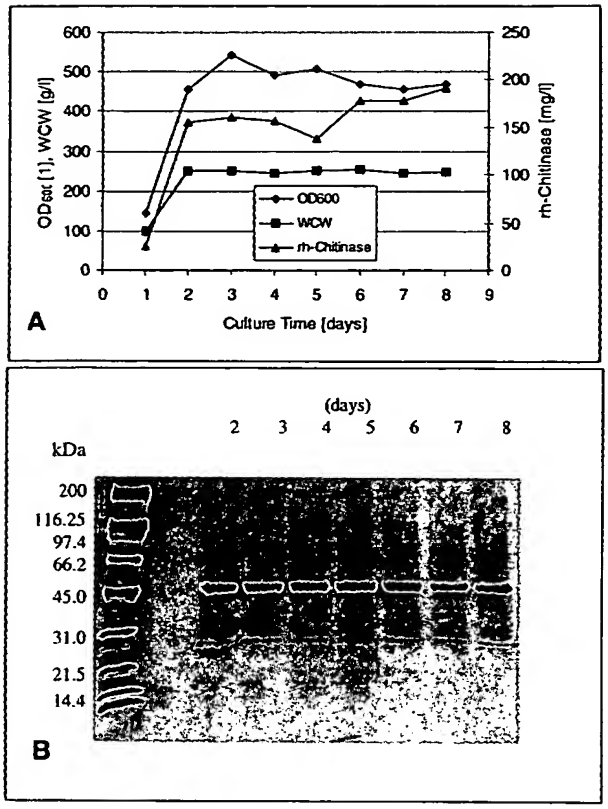

Figure 4. (A) Constitutive rh-chitinase expression in continuous culture with *P. pastoris* (host SMD 1168, *His*$^-$, vector pGAPZα). A 50% glycerol solution was fed (1.0 mL/min; 1.0 VVD). (B) SDS-PAGE. Lane 2–8: supernatant from continuous culture, days 2 to 8.

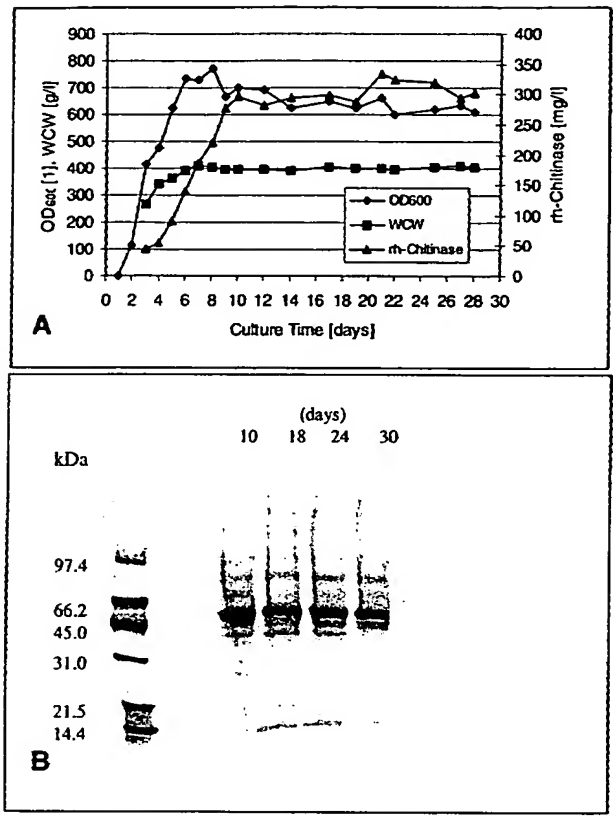

Figure 5. (A) Constitutive rh-chitinase expression in continuous culture with *P. pastoris* (host X33, vector pGAPZα). A 30% glucose continuous feed medium was fed (1.2 mL/min; 1.2 VVD). Culture was run successfully for 30 days. (B) SDS-PAGE. Lane 2–5: supernatant from continuous culture, days 10 to 30.

time period compared with rh-chitinase produced under fed-batch conditions. SDS-PAGE analysis of samples after day 8 was not performed because the onset of degradation typically occurred before day 8.

Continuous Fermentation of pGAPZα-X33 *His*$^+$ Clone

The highest producing clone was isolated when the X33 wild-type host was used for the transformation and colonies were selected on 2.0-mg/mL zeocin plates. This clone was grown in the continuous mode with an initial $D = 0.55$ day^{-1} or 0.8 VVD. The feeding rate was ramped up slowly to $D = 0.83$ day^{-1} or 1.2 VVD by day 6 (Fig. 5a). The rh-chitinase concentration increased steadily from about 50 mg/L to 300 mg/L within a period of 8 days. Cell yield plateaued on day 5 (~400 g/L WCW) and rh-chitinase concentration plateaued on day 9 (~300 mg/L). The culture was fed continuously with 30% glucose feed medium (see Materials and Methods) at $D =$; 0.83 day^{-1} or 1.2 VVD for an additional 24 days. The cell yield and rh-chitinase volumetric productivity remained steady at 400 g/L WCW and 360 mg/L per day, respectively.

As far as we know, this is the first report describing a *P. pastoris* high-cell-density fermentation continuing for 30 days. The culture showed no signs of decline for cell and product yields at run termination. SDS-PAGE analysis of samples indicated that the product was not degraded, even on day 30 of the fermentation (Fig. 5b). We have since cloned two other therapeutic proteins (one antiangiogenesis protein and one lysosomal enzyme) into *P. pastoris* with the *GAP* promoter and produced them using continuous fermentation conditions. Both recombinant proteins, which are normally digested by proteases under fed-batch conditions, were produced in an intact form using this continuous fermentation method (data not shown).

CONCLUSIONS

A process for the cultivation of *P. pastoris* in continuous fermentation, using the constitutive *GAP* promoter for the production of recombinant proteins, has been developed. To our knowledge, this is the first use of a continuous high-cell-density fermentation process employing the constitutive expression vector (pGAPZα) in *P. pastoris*. The productivity level achieved of 360 mg/L per day, or 3.6 mg/mg

DCW per day, was within the range seen in the literature for recombinant proteins produced in *P. pastoris.*

This continuous system provides not only for greatly enhanced production of recombinant proteins (approximately five- to sixfold higher productivity than fed-batch fermentation) and reduction of downtime associated with fermentor turnaround, but also for the production of intact proteins that are usually degraded in fed-batch fermentation. This may be due to the continual separation of sensitive proteins from the culture broth and/or a reduction in the level of protease(s) in the culture.

Also, the constitutive expression system allows for the safe handling of the *P. pastoris* production system, especially at the large scale, avoiding the use of methanol, which is flammable. This would greatly reduce the hazard and costs involved with large-scale production of recombinant therapeutic proteins in *P. pastoris* by alleviating the need for explosion-proof GMP facilities.

It is believed that this continuous *P. pastoris* expression system, employing the *GAP* promoter, is applicable to a wide range of proteins that previously could not be produced in the methylotrophic yeast *P. pastoris* due to proteolytic degradation and/or economic reasons.

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U.S. Patent No. 5,440,018, to Ohmura *et al.*

US005440018A

United States Patent [19]

Ohmura et al.

[11] Patent Number: 5,440,018

[45] Date of Patent: Aug. 8, 1995

[54] **RECOMBINANT HUMAN SERUM ALBUMIN, PROCESS FOR PRODUCING THE SAME AND PHARMACEUTICAL PREPARATION CONTAINING THE SAME**

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[73] Assignee: **The Green Cross Corporation**, Osaka, Japan

[21] Appl. No.: **36,387**

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[30] **Foreign Application Priority Data**

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Aug. 31, 1992	[JP]	Japan	4-232340
Sep. 22, 1992	[JP]	Japan	4-253142

[51] **Int. Cl.**[6] **C07K 14/765**; C07K 1/16; C07K 1/36; A61K 38/38

[52] **U.S. Cl.** **530/363**; 530/364; 530/412; 530/414; 530/415; 530/416

[58] **Field of Search** 530/363, 364, 412, 414, 530/415, 416; 514/8, 21

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Primary Examiner—William H. Beisner
Assistant Examiner—Nancy J. Degen
Attorney, Agent, or Firm—Sughrue, Mion, Zinn, Macpeak & Seas

[57] **ABSTRACT**

Human serum albumin obtained by gene manipulation techniques can be purified by a combination of specified steps in which a culture supernatant obtained from a human serum albumin-producing host is subjected to ultrafiltration, heat treatment, acid treatment and another ultrafiltration, followed by subsequent treatments with a cation exchanger, a hydrophobic chromatography carrier and an anion exchanger, and by salting-out to thereby obtain a pure form of human serum albumin which contains substantially no proteinous and polysaccharide contaminants, which is formulated into a pharmaceutical preparation. This process makes it possible to effeciently purify recombinant human serum albumin and to provide substantially pure human serum albumin which does not contain producer host-related substances and other contaminants and is sufficiently free from coloration.

10 Claims, 2 Drawing Sheets

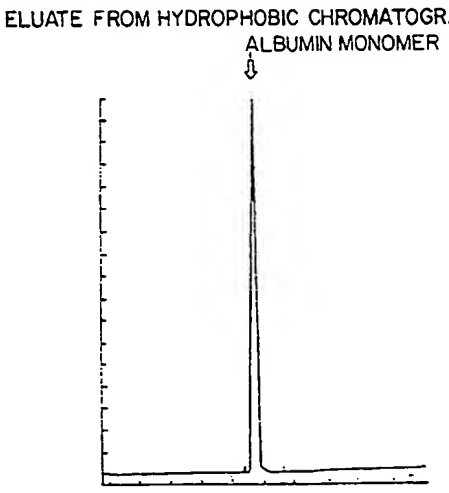


FIG. 1A

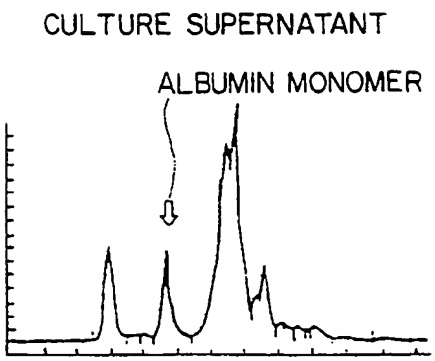

CULTURE SUPERNATANT
ALBUMIN MONOMER

FIG. 1B

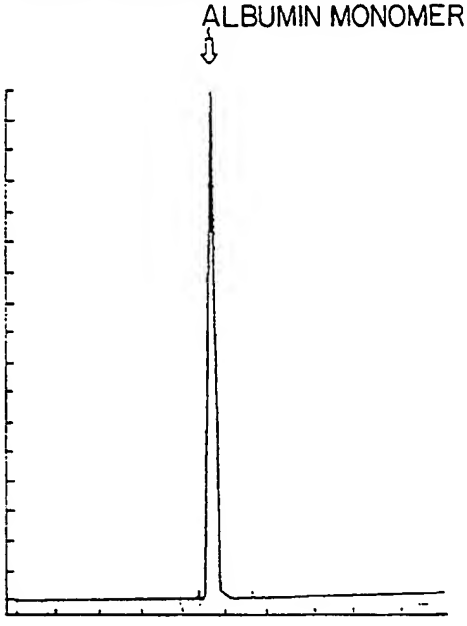
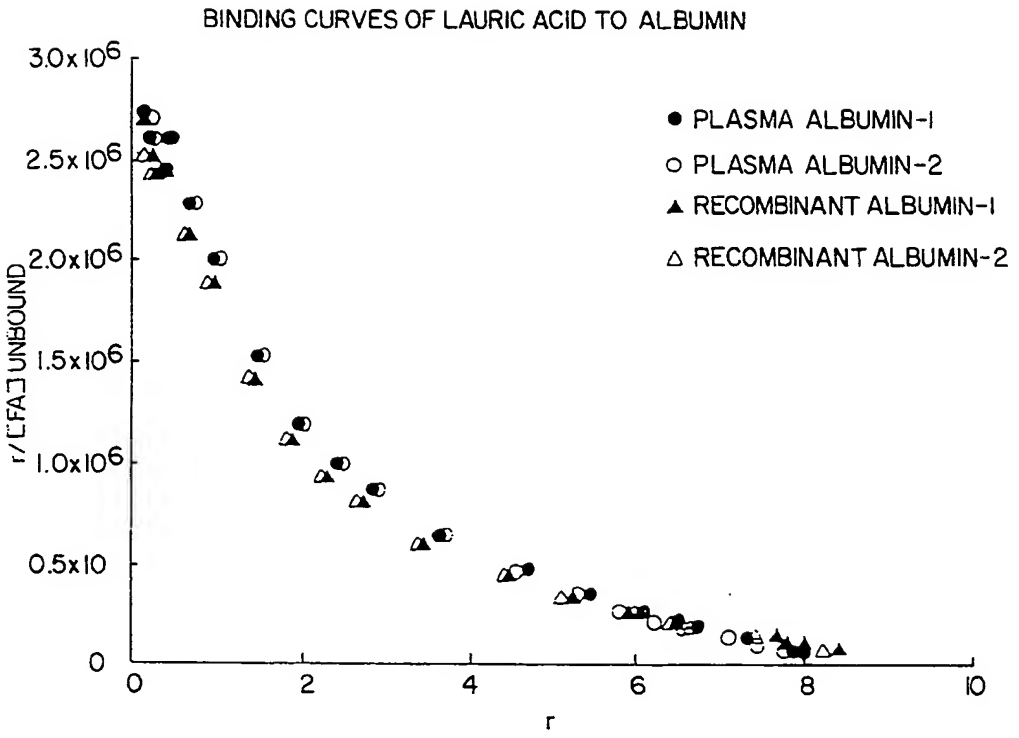

ELUATE FROM HYDROPHOBIC CHROMATOGRAPHY
ALBUMIN MONOMER

FIG. 2


BINDING CURVES OF LAURIC ACID TO ALBUMIN
3.0x10^6
2.5x10^6
2.0x10^6
1.5x10^6
1.0x10^6
0.5x10
0
r/[FA] UNBOUND
0
2
4
6
8
10
r
● PLASMA ALBUMIN-1
○ PLASMA ALBUMIN-2
▲ RECOMBINANT ALBUMIN-1
△ RECOMBINANT ALBUMIN-2

RECOMBINANT HUMAN SERUM ALBUMIN, PROCESS FOR PRODUCING THE SAME AND PHARMACEUTICAL PREPARATION CONTAINING THE SAME

FIELD OF THE INVENTION

The instant invention relates to a process for producing recombinant human serum albumin, in which the human serum albumin is purified through a combination of steps to yield a substantially pure form of human serum albumin.

BACKGROUND OF THE INVENTION

Albumin, especially human serum albumin (HSA), is an important protein of the circulatory system. The protein is produced in the liver and has a major role in maintaining normal osmotic pressure of body fluids, such as blood. It also serves as a carrier of various molecules.

HSA is administered under various clinical conditions. For example, in the case of shock or burn injury, it is necessary, in general, to administer HSA frequently to restore blood volume and to alleviate other injury-related symptoms. Patients suffering from hypoproteinemia and fetal erythroblastosis sometimes require HSA treatment.

In other words, a common indication for HSA administration is a loss of body fluids, such as during a surgical procedure, shock, burn injury or hypoproteinemia which causes edema.

Currently, HSA is produced mainly as a fractionated product of collected blood. Such a production process, however, has disadvantages in that it is not economical and the supply of blood is sporadic. In addition, collected blood sometimes contains undesirable substances, such as hepatitis virus. In consequence, it is profitable to develop a material which can be used as an HSA substitute.

Recent advances in recombinant DNA techniques have rendered possible microbial production of various types of useful polypeptides, and, as a result, a number of mammalian polypeptides already have been produced in various microorganisms. With regard to HSA, establishing techniques for the large scale production of HSA by recombinant methods and subsequent high grade purification also is in progress.

Techniques for the isolation and purification of HSA from plasma have been studied from various points of view and put into practical use. For example, the ethanol fractionation method of E. J. Cohn et al., PEG fractionation method, ammonium sulfate fractionation method and the like are well known methods. In addition to those methods, several purification processes recently have been developed, such as, for example, a process in which an anion exchanger treatment and a heat treatment at 60° C. for 10 hours are employed in combination (JP- A-2-191226 corresponding to EP-A-367220) and a process in which an anion exchanger treatment, a cation exchanger treatment and a heat treatment at 60° C. for 10 hours are employed in combination (JP-A-3-17123 corresponding to EP-A-428758). (The term "JP-A" as used herein means an "unexamined published Japanese patent application".)

However, in the case of producing HSA by means of gene manipulation techniques, it is highly probable that an HSA preparation of interest will be contaminated by certain coloring components which are contained in the raw materials or secreted by a microorganism during culturing of the host microorganism or are introduced during purification of the resulting HSA, and that those contaminants bind to HSA to cause coloring of the HSA itself. What is more, such contaminants cannot be removed sufficiently by means of any prior art process for the purification of plasma-derived HSA.

SUMMARY OF THE INVENTION

Taking the aforementioned problems involved in the prior art into consideration, the instant inventors have conducted intensive studies and, as a result, succeeded in developing a process for efficiently purifying HSA obtained by gene manipulation techniques.

An object of the instant invention is to provide human serum albumin obtained by means of gene manipulation techniques, which does not contain producer host-related substances or other contaminants and is substantially free from coloring.

More specifically, the instant invention provides a process for producing a recombinant human serum albumin comprising the steps of:

(1) treating a culture supernatant of a host which expresses human serum albumin, with a first ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 and then with a second ultrafiltration membrane having a molecular weight exclusive limit of from 1,000 to 50,000 to yield a first filtrate;

(2) heat-treating the first filtrate at 50° to 70° C. for 30 minutes to 5 hours to yield a heated sample;

(3) acid-treating the heated sample at a pH of 3 to 5 to yield an acid-treated sample;

(4) treating the acid-treated sample using an ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 to yield a second filtrate;

(5) allowing the second filtrate to contact with a cation exchanger at a pH of 3 to 5 and a salt concentration of 0.01 to 0.2M, and then exposing the cation exchanger to a pH of 8 to 10 and a salt concentration of 0.2 to 0.5M to yield a first eluate;

(6) allowing the first eluate to contact with a carrier for hydrophobic chromatography at a pH of 6 to 8 and a salt concentration of 0.01 to 0.5M, and recovering non-adsorbed fractions to yield a second eluate; and

(7) allowing the second eluate to contact with an anion exchanger at a pH of 6 to 8 and recovering non-adsorbed fractions to yield the albumin.

An additional step (8) may be employed in the above process, in which the resulting eluate of step (7) further is allowed to contact with a chelate resin and the resulting non-adsorbed fractions are recovered.

Another object of the instant invention is to provide a substantially pure recombinant human serum albumin, wherein a 25% solution of the albumin contains contaminated proteins in an amount of 0.1 ng/ml or less and contaminated polysaccharides in an amount of 1 ng/ml or less.

Another object of the instant invention is to provide a pharmaceutical preparation comprising recombinant human serum albumin, acetyltryptophan or a salt thereof and sodium caprylate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(*a*) and (*b*) are graphs showing the results of HPLC analysis of HSA obtained as the culture super-

nates and after the hydrophobic chromatography purification step.

FIG. 2 is a graph showing binding curves of lauric acid to HSA.

DETAILED DESCRIPTION OF THE INVENTION

The term "substantially pure HSA" used herein means that a 25% aqueous solution of purified HSA contains contaminated proteins and polysaccharides in an amount of at most 0.1 ng/ml or below and 1 ng/ml or below, respectively, or that the purity of purified HSA is 99.999999% or more.

In the process of the instant invention, the step (6) may be replaced by another step (6) in which the resulting eluate of the step (5) is allowed to contact with a carrier for hydrophobic chromatography at a pH of 6 to 8 and a salt concentration of 1 to 3M, and then the carrier is exposed to a pH of 6 to 8 and a salt concentration of 0.01 to 0.5M.

Also, the step (7) of the process of the present invention may be replaced by another step (7) in which the resulting eluate of the step (6) is allowed to contact with an anion exchanger at a pH of 6 to 8 and a salt concentration of 0.001 to 0.05M, and then the anion exchanger is exposed to a pH of 6 to 8 and a salt concentration of 0.05 to 1M.

In addition, the process of the present invention may further comprise a salt precipitation (salting-out) step following step (5), step (6) or step (7), in which the salt precipitation is carried out by exposing the first eluate, the second eluate or the albumin to a pH of 3 to 5 and a salt concentration of 0.5 to 3M to yield a precipitation and dissolving the precipitate in a buffer.

The instant invention is described in detail below.

(1) Recombinant HSA

The HSA-producing host prepared by means of gene manipulation techniques to be used in the instant invention is not limited so long as the HSA has been prepared via gene manipulation techniques, hence the host can be selected from hosts already known in the art, as well as those hosts which will be developed in the future. Illustrative examples of the host include microbial cells, such as *Escherichia coli,* various yeast species, *Bacillus subtilis* and the like, and animal cells. Particularly preferred hosts are yeast species, especially those belonging to the genus Saccharomyces, such as *Saccharomyces cerevisiae,* or the genus Pichia, such as *Pichia pastoris.* Auxotrophic strains or antibiotic-sensitive strains also may be used. *Saccharomyces cerevisiae* AH22 (a, his 4, leu 2, can 1), Pichia pastoris GTS115 (his 4) and the like strains are used preferably.

Preparation of the HSA-producing hosts, production of HSA by culturing the hosts and isolation and recovery of HSA from the resulting culture broth may be effected using known techniques or modified procedures thereof. For example, preparation of an HSA-producing host (or an HSA-producing strain) may be effected using a process in which a natural human serum albumin gene is used (JP-A-58-56684 corresponding to EP-A-73646, JP-A-58-90515 corresponding to EP-A-79739 and JP-A-58-150517 corresponding to EP-A-91527), a process in which a modified human serum albumin gene is used (JP-A-62-29985 and JP-A-1-98486 corresponding to EP-A-206733), a process in which a synthetic signal sequence is used (JP-A-1-240191 corresponding to EP-A-329127), a process in which a serum albumin signal sequence is used (JP-A-2-167095 corresponding to EP-A-319641), a process in which a recombinant plasmid is introduced into chromosome (JP-A-3-72889 corresponding to EP-A-399455), a process in which hosts are fused (JP-A-3-53877 corresponding to EP-A-409156), a process in which mutation is generated in a methanol containing medium, a process in which a mutant AOX2 promoter is used (EP-A-506040), a process in which HSA is expressed in *B. subtilis* (JP-A-62-215393 corresponding to EP-A-229712), a process in which HSA is expressed in yeast (JP-A-60-41487 corresponding to EP-A-123544, JP-A-63-39576 corresponding to EP-A-248657 and JP-A-63-74493 corresponding to EP-A-251744) and a process in which HSA is expressed in Pichia (JP-A-2-104290 corresponding to EP-A-344459).

The process in which mutation is generated in a methanol-containing medium is carried out in the following manner.

Firstly, a plasmid containing a transcription unit which is constructed so as to express HSA under the control of AOX1 promoter is introduced into the AOX1 gene region of an appropriate host, preferably a Pichia yeast, more preferably Pichia strain GTS115 (NRRL deposition number Y-15851) (JP-A-2-104290 corresponding to EP-A-344459) to obtain a transformant. Since the thus obtained transformant does not grow well in a methanol-containing medium, mutation of the transformant is effected by culturing the transformant in a methanol-containing medium to isolate a mutant strain which is capable of growing in the medium. The methanol concentration in the medium may be in the range of approximately from 0.01 to 5%. The medium may be either synthetic or natural, and the culturing may be carried out at 15° to 40° C. for 1 to 1,000 hours.

Culturing of an HSA-producing host (an HSA production process) may be carried out using known processes disclosed in the aforementioned references, or in accordance with a process disclosed in JP-A-3-83595 in which high concentration substrate inhibition of HSA producer cells is avoided by gradually adding a high concentration glucose solution to a medium by means of fed batch fermentation, thereby enabling production of both the producer cells and the product in high concentrations, or in accordance with another process disclosed in JP-A-4-293495 corresponding to EP-A-504823 in which productivity of HSA is improved by adding fatty acids to a medium.

Isolation and recovery of HSA may be carried out using known processes disclosed in the aforementioned references, or in accordance with a process disclosed in JP-A-3-103188 corresponding to EP-A-420007 in which proteases are inactivated by heat treatment or a coloration inhibition process disclosed in JP-A-4-54198 corresponding to U.S. Pat. No. 5,132,404 or EP-A464590 in which HSA is separated from coloring substances using at least one adsorbent selected from the group consisting of anion exchangers, hydrophobic carriers and activated charcoal.

The medium for culturing a transformed host may be prepared by adding fatty acids having 10 to 26 carbon atoms, or salts thereof, to a known medium, and culturing the transformant under known conditions. The medium may be either synthetic or natural, but preferably a liquid medium. For example, a suitable synthetic medium may be composed of: carbon sources, such as various saccharides and the like; nitrogen sources, such as urea, ammonium salts, nitrates and the like; trace

nutrients, such as various vitamins, nucleotides and the like; and inorganic salts, such as of Mg, Ca, Fe, Na, K, Mn, Co, Cu and the like. An illustrative example of such a medium is YNB liquid medium which consists of 0.7% Yeast Nitrogen Base (Difco) and 2% glucose. An illustrative example of a useful natural medium is YPD liquid medium which consists of 1% Yeast Extract (Difco), 2% Bacto Peptone (Difco) and 2% glucose. The medium pH may be neutral, weakly basic or weakly acidic. In the case of a methanol assimilating host, the medium may be further supplemented with methanol in an amount of approximately from 0.01 to 5%.

Culturing of a host may be carried out preferably at 15° to 43° C. (20 to 30° C. for yeast strains, 20° to 37° C. for bacterial strains) for 1 to 1,000 hours, by means of static or shaking culturing or batch, semi-batch or continuous culturing under agitation and aeration.

In that instance, it is desirable to prepare a seed culture prior to the batch culturing. The seed culturing may be carried out using the aforementioned YNB liquid medium or YPD liquid medium, preferably at 30° C. (yeast) or 37° C. (bacterium) and for 10 to 100 hours.

After completion of the culturing, HSA is recovered from the resulting culture medium or cells in the usual way.

(2) Purification of HSA

(i) Ultrafiltration

High molecular weight substances other than HSA, as well as low molecular weight substances, are separated and removed from a culture supernatant obtained after separation HSA-producing host cells, using ultrafiltration techniques.

High molecular weight substances are removed using an ultrafiltration membrane having a molecular weight exclusive limit of approximately from 100,000 to 500,000, preferably around 300,000, and low molecular weight substances are removed using another ultrafiltration membrane having a molecular weight exclusive limit of approximately from 1,000 to 50,000, preferably from about 10,000 to about 30,000.

Separation of remaining HSA-producing host cells is effected simultaneously at the time of the removal of high molecular weight substances, and concentration of the liquid is effected at the time of the removal of low molecular weight substances.

(ii) Heat treatment

The concentrated solution obtained in the above step (i) is subjected to heat treatment at 50° to 70° C. for approximately 30 minutes to 5 hours, preferably at 60° C. for approximately 1 to 3 hours.

Preferably, the heating is conducted in the presence of a stabilizing agent. Preferred examples of the stabilizer include acetyltryptophan and an organic carboxylic acid having 6 to 18 carbon atoms, or a salt thereof. The stabilizers may be used in combination. Acetyltryptophan may be used in an amount of approximately from 1 to 100 mM. Illustrative examples of the organic carboxylic acid having 6 to 18 carbon atoms include caproic acid (6 carbon atoms), caprylic acid (8 carbon atoms), capric acid (10 carbon atoms), lauric acid (12 carbon atoms), palmitic acid (16 carbon atoms), oleic acid (18 carbon atoms) and the like. Illustrative examples of the salts include alkali metal salts such as sodium salt, potassium salt and the like, and alkaline earth metal salts, such as calcium salt and the like. The organic carboxylic acid having 6 to 18 carbon atoms or a salt thereof may be used in an amount of approximately from 1 to 100 mM.

In the heat treatment step, color development caused by the heating can be prevented by the addition of a thiol compound (for example, mercaptoethanol, cysteine, reduced glutathione or the like) in an amount of approximately from 1 to 100mM, preferably from 5 to 30mM, and aminoguanidine in an amount of from 10 to 1,000 mM. A part of this step has already been disclosed in JP-A-3-103188.

(iii) Acid treatment

The heat-treated solution of the above step (ii) is adjusted to a pH of approximately 3 to 5, preferably 4 to 4.6, with acid and then allowed to stand for a period of approximately 1 to 12 hours. Examples of the acid include acetic acid, hydrochloric acid, phophoric acid, sulfuric acid and the like.

(iv) Ultrafiltration

In this step, polymerized high molecular weight contaminants are removed by ultrafiltration. High molecular weight substances are removed using an ultrafiltration membrane having a molecular weight exclusive limit of approximately from 100,000 to 500,000, preferably around 300,000. If necessary, buffer exchange may be carried out for use in the following cation exchanger treatment, using an ultrafiltration membrane having a molecular weight exclusive limit of approximately from 1,000 to 50,000, preferably from about 10,000 to about 30,000.

(v) Cation exchanger treatment

Usable as cation exchangers are those having sulfo groups, carboxyl groups and the like. Illustrative examples of the sulfo group-containing cation exchangers include sulfoagarose (trade name, S-Sepharose ®, available from Pharmacia), sulfopropyl-dextran (trade name, SP-Sephadex ®, available from Pharmacia), sulfopropyl-polyvinyl (trade name, SP-Toyopearl ®, available from Tosoh Corp.) and the like. Illustrative examples of the carboxyl group-containing cation exchangers include carboxymethyl-dextran (trade name, CM-Sephadex ®, available from Pharmacia; and trade name, CM-Cellulofine ®, available from Seikagaku corp.) and the like.

The exchanger can be equilibrated with an appropriate buffer such as acetate buffer having a pH of approximately 3 to 5, preferably 4 to 4.6 and containing a salt such as sodium chloride in a concentration of approximately 0.01 to 0.2M, preferably 0.05 to 0.1M. The same buffer can be used for contacting and washing treatments. Elution may be effected with an appropriate buffer such as phosphate buffer having a pH of generally 8 to 10, preferably 8.5 to 9.5 and containing a salt such as sodium chloride in a concentration of generally 0.2 to 0.5M, preferably 0.3 to 0.4M.

(vi) Hydrophobic chromatography

Carriers for use in hydrophobic chromatography include those containing an alkyl group (butyl group, octyl group, octyldecyl group and the like), each group having 4 to 18 carbon atoms, and those containing a phenyl group. Illustrative examples of the butyl group-containing carriers include butyl-agarose, butyl-polyvinyl (trade name, Butyl Toyopearl ®, available from Tosoh Corp.) and the like, those of the octyl group-containing and octyldecyl group-containing carriers include octyl-agarose and octyldecyl-agarose, respectively, and those of the phenyl group-containing carrier include phenyl-cellulose (trade name, Phenyl Cellulofine ®, available from Seikagaku Corp.) and the like.

In this step, HSA can be recovered from non-adsorbed fractions. In that case, contacting may be effected using an appropriate buffer such as phosphate buffer having a pH of approximately 6 to 8, preferably 6.5 to 7 and containing a salt such as sodium chloride in a concentration of approximately 0.01 to 0.5M, preferably 0.05 to 0.2M.

HSA also can be recovered by elution after adsorption to the aforementioned carrier. In that case, contacting and washing may be carried out using an appropriate buffer such as phosphate buffer having a pH of approximately 6 to 8, preferably from 6.5 to 7 and containing a salt such as sodium chloride in a concentration of approximately 1 to 3M, preferably 1.5 to 2M. The elution may be effected with an appropriate buffer such as phosphate buffer having a pH of approximately 6 to 8, preferably 6.5 to 7 and containing a salt such as sodium chloride in a concentration of approximately 0.01 to 0.5M, preferably 0.05 to 0.2M.

(vii) Anion exchanger treatment

Examples of anion exchangers include those containing the diethylaminoethyl (DEAE) group, those containing a quaternary aminoethyl (QAE) group and the like. Illustrative examples of the DEAE group-containing anion exchangers include DEAE-agarose (trade name, DEAE-Sepharose ®, available from Pharmacia), DEAE-dextran (trade name, DEAE-Sephadex ®, available from Pharmacia), DEAE-polyvinyl (trade name, DEAE-Toyopearl ®, available from Tosoh Corp.) and the like. Illustrative examples of the QAE group-containing anion exchangers include QAE-agarose (trade name, Q-Sepharose ®, available from Pharmacia), QAE-polyvinyl (trade name, QAE-Toyopearl ®, available from Tosoh Corp.) and the like.

In this step, HSA can be recovered from non-adsorbed fractions. In that case, contacting may be effected using an appropriate buffer such as phosphate buffer having a pH of approximately 6 to 8, preferably 6.5 to 7 and a salt concentration of approximately 0.01 to 0.1M.

HSA also can be recovered by elution after adsorption to the aforementioned carrier. In that case, contacting and washing may be carried out using the same buffer as described above except for containing a salt such as sodium chloride in a concentration of 0.001 to 0.05M and elution may be carried out with the same buffer but having a salt concentration of 0.05 to 1M.

(viii) Salting-out

In this step, HSA is precipitated specifically by adding a salt component to the sample solution to a final salt concentration of approximately 0.1 to 3M, preferably 0.5 to 1.5M and then adjusting the resulting solution to a pH of approximately 3 to 5, preferably 3.5 to 4.5. Impurities in the supernatant fluid are removed by separating the HSA precipitate.

The thus precipitated HSA is dissolved in an appropriate buffer solution. Though not particularly limited, usable as salt components to adjust ionic strength are sodium chloride, potassium chloride, ammonium sulfate, sodium (or potassium) thiocyanate, sodium sulfate and the like. Also, though not particularly limited, separation of the precipitated HSA from the supernatant fluid may be effected preferably by centrifugation, press separation, cross-flow membrane separation and the like.

This step may be carried out preferably after the anion exchanger treatment step (vii), but may also be interposed between the cation exchanger treatment step (v) and the hydrophobic chromatography treatment step (vi) or between the hydrophobic chromatography treatment step (vi) and the anion exchanger treatment step (vii).

(ix) Chelate resin treatment

The above purification steps may further contain a step of allowing HSA to contact with a chelate resin which has a specified ligand moiety. This step may be carried out preferably after the anion exchanger treatment or salting-out precipitation treatment, whichever is the later.

Preferably, the carrier moiety of the chelate resin may have hydrophobic nature. Examples of such a type of carrier moiety include a copolymer of styrene and divinylbenzene, a copolymer of acrylic acid and methacrylic acid and the like.

Examples of the ligand moiety include a thiourea group, as well as a polyamine group (including a polyalkylene polyamine group such as polyethylene polyamine or the like) which contains, in one molecule, a plurality of sub-groups consisting of a polyol group such as an N-methylglucamine group, an imino group, an amino group, an ethyleneimino group and the like. Illustrative examples of preferred commercially available chelate resins having the above-described carrier and ligand moieties, include DIAION CRB02 ® (ligand moiety, N-methylglucamine group, available from Mitsubishi Kasei Corp.), DIAION CR20 ® (ligand moiety, $—NH(CH_2CH_2NH)_nH$, available from Mitsubishi Kasei Corp.), LEWATIT TP214 ® (ligand moiety, $—NHCSNH_2$, available from Bayer) and AMBERLITE CG4000 ®, all of which have a copolymer of styrene and divinylbenzene as the carrier moiety.

Preferred conditions for the chelate resin treatment are as follows.

pH: acidic or neutral (pH 3 to 9, preferably 4 to 7), period: at least 1 hour, preferably 6 hours or more, ionic strength: 50 mmho or less, preferably 1 to 10 mmho,

mixing ratio: 0.1 to 100 g, preferably 1 to 10 g, of the resin based on 250 mg of HSA (wet basis).

In the process, contaminated coloring substances derived from the raw material or the host are adsorbed to the chelate resin, thus enabling reduction of coloration of HSA.

The steps (v), (vi), (vii) and (ix) may be carried out using a column or in a batchwise operation, with the use of a column being preferred.

HSA thus purified through the above steps (i) to (vii) and the additional salting-out and chelate resin treatment steps is substantially free from coloring, which means a coloring degree of the purified HSA ranges approximately from 0.001 to 0.005 in terms of an $A_{500\ nm}/A_{280\ nm}$ ratio in a 25% HSA solution. The term "a 25% HSA solution" used herein means a solution of 25% protein which may contain contaminated proteins in an amount lower than the detection limit, namely 0.1 ng/ml, other than purified HSA. Coloration of HSA is reduced to a level of from ½ to 1/10 by the chelate resin treatment of the instant invention. Especially, absorbance at around 500 nm, namely reddish coloration, is reduced to a level of from ⅓ to 1/10.

In addition, fatty acids which adsorb or bind to HSA, derived from the medium or the host or secreted by the host, can be removed by the chelate resin treatment.

Amounts of fatty acids adsorbed to HSA which has been purified through the above steps (i) to (vii) and the additional salting-out step are reduced to a level of 1/10

or below, preferably 1/100 or below, by the chelate resin treatment.

Amounts of fatty acids adsorbed to HSA can be measured in accordance with a generally used means such as Duncombe's extraction method (Clin. Chim. Acta., 9, 122–125 (1964)) or acyl-CoA synthetase (ACS)-acyl-CoA oxidase (ACOD) method in which ACS and ACOD are used.

The Duncombe's extraction method comprises, in principle, converting fatty acids into copper salts using a copper reagent, extracting with chloroform and then subjecting the extract to color development with Bathocuproin. The method can be carried out easily using a kit such as NEFA-Test Wako ® (Wako Pure Chemical Industries, Ltd.) which contains Bathocuproin.

On the other hand, the ACS-ACOD method comprises, in principle, reacting fatty acids with acyl-CoA synthetase and acyl-CoA oxidase to generate H_2O_2 and exposing the thus-formed H_2O_2 by a color development scheme using oxidation-condensation reaction of a chromogenic substance in the presence of peroxidase. That method also can be carried out easily using a measuring kit such as NEFAC-Test Wako ® (Wako Pure Chemical Industries, Ltd.).

Substances to be removed by the defatting method of the present invention are fatty acids and esters thereof which are derived from raw materials for HSA production, such as that derived from blood, a medium, a host or those secreted by the host.

Examples of fatty acids to be removed include saturated fatty acids having 8 to 20 carbon atoms, such as palmitic acid, stearic acid and the like, and unsaturated fatty acids having 16 to 20 carbon atoms, such as oleic acid, linoleic acid, arachidonic acid and the like.

Since this step is effective for the removal of those fatty acids, it can be applied to the defatting of HSA molecules to which those fatty acids are attached, independent of the origin of the HSA.

(4) Pharmaceutical preparation

The HSA thus obtained may be made into pharmaceutical preparations by generally known means such as 10 hours of heat sterilization at 60° C., ultrafiltration, filter sterilization, dispensation, freeze-drying and the like. An illustrative example of the pharmaceutical preparation of the present invention is a liquid preparation which contains HSA in an amount of 5 to 25%, has a pH of approximately 6.4 to 7.4 and has an osmotic pressure ratio of around 1.

The HSA-containing pharmaceutical preparation of the instant invention may contain stabilizers which include acetyltryptophan or a salt thereof (e.g., sodium salt) and sodium caprylate. Each stabilizer may be used in an amount of approximately 0.001 to 0.2M, preferably 0.01 to 0.05M in a 25% HSA solution. The sodium content may be 3.7 mg/ml or less. The HSA preparation may further contain pharmaceutically acceptable additives such as sodium chloride and the like.

In general, the stabilizers may be added prior to the aforementioned preparation steps such as 10 hours of heat sterilization at 60° C., ultrafiltration, filter sterilization, dispensation, freeze-drying and the like. Therefore, not only preservation stability of HSA but also its stability during the preparation process of the pharmaceutical preparation of the instant invention can be improved.

The HSA-containing pharmaceutical preparation thus obtained can be used clinically as injections in the same manner as the case of the prior art plasma-derived HSA preparations. For example, it may be used for the purpose of rapidly increasing blood volume, mainly at the time of shock, supplementing circulation blood volume, improving hypoproteinemia or maintaining collagen osmotic pressure. More illustratively, the HSA-containing pharmaceutical preparation of the present invention can be used effectively for the treatment of hypoalbuminemia caused by the loss of albumin (burn injury, nephrotic syndrome or the like) or by the reduction of albumin synthesizing ability (hepatic cirrhosis or the like), as well as for the treatment of hemorrhagic shock and the like.

The pharmaceutical preparation may be administered gradually by intravenous injection or intravenous drip infusion, with a dose of generally from 20 to 50 ml as a 25% HSA solution (5 to 12.5 g as HSA) per one administration for an adult. The dose may be changed optionally depending on the age, symptoms, weight and the like of the patient. Properties of the purified recombinant HSA.

(5) Purified HSA

The HSA of the instant invention is a homogeneous substance having a molecular weight of about 67,000 and an isoelectric point of 4.6 to 5.0. The HSA consists of a monomer and contains substantially no dimers, polymers or decomposed products. In fact, the total content of dimers, polymers and hydrolyzed products is approximately 0.01% or less.

Also, the HSA of the instant invention contains substantially no producer host-derived contaminants, such as protein, polysaccharide and the like, which means contaminants having antigenecity detectable by immunoassay such as EIA, RIA, PHA and so forth. Thus, the HSA of the instant invention contains substantially no host-derived contaminants having antigenecity detectable by immunoassay. In the case of a 25% HSA solution, the protein content may be 1 ng/ml or below, preferably 0.1 ng/ml or below, and the polysaccharide content may be 10 ng/ml or below, preferably 1 ng/ml or below. In that case, the purity of the HSA is calculated to be 99.999999% or more, preferably 99.9999999% or more.

The degree of coloring of the 25% HSA solution may be in the range of from 0.01 to 0.05 in terms of an A_{350}/A_{280} ratio, from 0.001 to 0.02 as an A_{450}/A_{280} ratio and from 0.001 to 0.005 as an A_{500}/A_{280} ratio.

In addition, the amount of fatty acids linked to the HSA may be one molecule or less, preferably 0.1 molecule or less, per one HSA molecule.

According to the instant invention, recombinant HSA can be purified efficiently. In addition, the instant invention can provide substantially pure recombinant HSA which does not contain producer host-related substances and other contaminants, and is sufficiently free from coloration.

The following examples are provided to further illustrate the instant invention. It is to be understood, however, that the examples are not to limit the scope of the present invention.

REFERENCE EXAMPLE 1

(1) Used strain, *Pichia pastoris* GCP101

A strain of *Pichia pastoris*, PC4130, obtained in accordance with the process disclosed in JP-A-2-104290, was made by digesting a plasmid pPGP1, containing a transcription unit which is constructed so as to express HSA under the control of an AOX1 promoter, with NcoI and then substituting the resulting NotI-digested fragment

for the AOX1 gene region of a Pichia pastoris strain GTS115 (his4). The strain does not grow well in a medium containing methanol as the carbon source (Mut− strain) because of the deletion of the AOX1 gene.

The strain PC4130 was inoculated into 3 ml of YPD medium (1% yeast extract, 2% Bacto Peptone and 2% glucose). After 24 hours of culturing, the cells were inoculated into 50 ml of YPD medium so that the cell density should be adjusted to initial turbidity with an OD_{540} of 0.1. After 3 days of culturing at 30° C., the resulting cells again were inoculated into 50 ml of YPD medium at an initial cell turbidity of 0.1 at OD_{540}. Thereafter, subculturing was repeated every 3 days in the same manner. After each subculturing, cells were diluted with sterile water and poured onto a 2% MeOH—YNBw/oa.a. plate (0.7% Yeast Nitrogen Base without Amino Acids, 2% methanol and 1.5% agar powder) in an inoculum size of 10^7 cells/plate, followed by 5 days of culturing at 30° C. to judge the present/absence of colonies. Twenty colonies Were found on the 2% MeOH—YNBw/oa.a. plate after 12 days of the successive subculturing. Mut− strains can hardly grow on the 2% MeOH—YNBw/oa.a. medium while Mut+ strains can grow well. That is, advent of a colony means that the strain acquired the capacity of increased methanol assimilation and thus a Mut+ strain was obtained. One of the thus obtained colonies was diluted appropriately with sterile water and spread onto a 2% MeOH—YNBw/oa.a. plate to isolate single colonies. One of the resulting single colonies was named GCP101.

(2) Culturing of the strain

(First seed culture)

A 1 ml portion of the strain which had been frozen in glycerol was inoculated into a 1,000 ml baffled Erlenmeyer flask containing 200 ml of YPD medium (see Table 1) and cultured at 30° C. for 24 hours with shaking.

TABLE 1

Composition of YPD medium	
Components	Concentration (g/L)
Yeast extract	10
Peptone	20
Glucose	20

(Second seed culture)

The first seed culture broth was inoculated into a 10 liter-jar fermentor containing 5 liters of YPD medium, and the second seed culturing was carried out at 30° C. for 24 hours with agitation and at an aeration rate of 5 liters per minutes. In the seed culturing, the pH of the medium was not controlled.

(Main culture)

The second seed culture broth was transferred into a 1,200 liter-fermentor containing 250 liters of a batch culture medium (see Table 2), and batch culturing was started with agitation and aeration under an internal pressure of 0.5 kg/cm^2 and at a maximum aeration rate of 800 liter/min under atmospheric pressure. The agitation rate was controlled so that the level of dissolved oxygen in the medium was maintained at approximately 50 to 30% of the saturated dissolved oxygen concentration. When the glycerol in the batch culture medium was consumed, addition of a feeding medium (see Table 3) was started. Feeding rate of the medium was controlled using a computer in such a manner that methanol did not accumulate in the culture medium, thereby effecting a high density culturing. The medium pH was controlled at a fixed level of 5.85 by the addition of 28% aqueous ammonia. For defoamation of the culture medium, an antifoam agent (Adecanol ®, manufactured by Asahi Denka Kogyo K.K.) was added in an amount of 0.30 ml/liter at the time of the commencement of the batch culture, thereafter adding a small amount when required.

TABLE 2

Composition of batch culture medium	
Components	Amount per liter
Glycerol	50.0 g
H_3PO_4 (85%)	14.0 ml
$CaSO_4.2H_2O$	0.6 g
K_2SO_4	9.5 g
$MgSO_4.7H_2O$	7.8 g
KOH	2.6 g
Biotin solution *1	1.6 ml
YTM solution *2	4.4 ml

*1 Biotin solution: 0.2 g/l

*2 YTM solution:

Components	Amount per liter
$FeSO_4.7H_2O$	65.0 g
$CuSO_4.5H_2O$	6.0 g
$ZnSO_4.7H_2O$	20.0 g
$MnSO_4.4\text{-}5H_2O$	3.0 g
H_2SO_4	5.0 ml

TABLE 3

Composition of feeding medium	
Components	Amount
YTM solution	2 ml
Methanol	1,000 ml

REFERENCE EXAMPLE 2

An HSA expression plasmid pMM042 was constructed using an AOX2 promoter (a mutant of the natural AOX2 promoter (YEAST, 5, 167–177, 1988; Mol. Cell. Biol., 9, 1316–1323, 1989), in which the 255th base upstream from the initiation codon of said promoter is changed from T to C) isolated from the strain GCP101 obtained in Reference Example 1. The thus constructed plasmid was introduced into *Pichia pastoris* GTS115 to obtain a transformant UHG42-3 (EP-A-506040). Thereafter, the thus obtained transformant was cultured in accordance with the procedure of Reference Example 1, thereby allowing the transformant to produce HSA.

EXAMPLE 1

[i] Isolation of Culture Supernatant—Membrane Fractions (I) and (II)

About an 800 liter portion of the culture broth obtained in Reference Example 1 was subjected to a filter press to isolate the culture supernatant. The resulting supernatant subsequently was treated with an ultrafiltration membrane having a molecular weight exclusive limit of 300,000. Then, the resulting filtrate was concentrated to a volume of about 80 liters using an ultrafiltration membrane having a molecular weight exclusive limit of 30,000 [membrane fraction (I)].

Next, the membrane fraction (I) was heat-treated at 60° C. for 3 hours in the presence of 5 mM of sodium caprylate, 10 mM of cysteine and 100 mM of aminoguanidine at pH 7.5. The thus heat-treated solution was cooled down rapidly to about 15° C., adjusted to pH 4.5 and then treated with an ultrafiltration membrane having a molecular weight exclusive limit of

300,000 [membrane fraction (II)]. Thereafter, using an ultrafiltration membrane having a molecular weight exclusive limit of 30,000, the buffer in the resulting albumin solution was replaced by a 50 mM acetate buffer (pH 4.5) containing 50 mM of sodium chloride.

[ii] Cation Exchanger Treatment

The albumin solution obtained in the above step [i] was applied to a column packed with S-Sepharose ® which had been equilibrated in advance with a 50 mM acetate buffer (pH 4.5) containing 50 mM of sodium chloride, the column was washed thoroughly with the same buffer and then elution was carried out with a 0.1M phosphate buffer (pH 9) containing 0.3M sodium chloride.

Polysaccharide content before and after the cation exchanger treatment was measured in accordance with the phenolsulfuric acid method to find that the polysaccharide content has been reduced by 1/20 by this treatment.

[iii] Hydrophobic Chromatography

The albumin solution eluted from the S-Sepharose ® column was applied to a column packed with Phenyl Cellulofine ® which has been equilibrated in advance with a 50 mM phosphate buffer (pH 6.8) containing 0.15M sodium chloride. Since albumin does not adsorb to Phenyl Cellulofine ® under such conditions, the albumin fractions which passed through the column were collected.

The albumin solution thus recovered was concentrated to a volume of about 50 liters using an ultrafiltration membrane having a molecular weight exclusive limit of 30,000, and at the same time, the buffer in the albumin solution was replaced by a 50 mM phosphate buffer (pH 6.8).

[iv] Anion Exchanger Treatment

The albumin solution thus treated with hydrophobic chromatography, concentrated and buffer-exchanged in the above step [iii] was applied to a column packed with DEAE-Sepharose ® which had been equilibrated in advance with a 50 mM phosphate buffer (pH 6.8). Under such conditions, albumin was not adsorbed to the DEAE-Sepharose ® but passed through the column.

[v] Salting-Out of HSA

To a 5% HSA solution was added sodium chloride to a final concentration of 1M. The resulting solution was adjusted to pH 3.5 with acetic acid to precipitate HSA, and the thus precipitated HSA was separated from the supernatant fluid by centrifugation, thereby effecting removal of impurities. The albumin precipitate can be used as an injection by dissolving it in a liquid, concentrating and buffer-exchanging the thus dissolved solution using an ultrafiltration membrane having a molecular weight exclusive limit of 30,000, adding a stabilizing agent to the thus treated solution if necessary, and then subjecting the resulting solution to filter sterilization.

[vi] HPLC Analysis of Purified HSA

The HSA preparation obtained after the purification step by hydrophobic chromatography was analyzed by means of HPLC gel filtration under the following conditions.

(a) Column: TSK gel G3000SWxL ® (Tosoh Corp.)
(b) Eluent: 0.3M NaCl/50 mM phosphate buffer
(c) Detection: absorbance at 280 nm

As shown in FIG. 1, the purified HSA preparation was found as a single peak of HSA monomer.

[vii] Analysis of Yeast-Derived Components

A culture supernatant of a yeast strain which does not produce albumin was partially purified in accordance with the purification process of the instant invention, and separated into a protein fraction and a polysaccharide fraction. Rabbits were immunized with the protein fraction or the polysaccharide fraction. Using an antiserum preparation obtained in that manner, detection of yeast-derived components in the purified albumin solution was carried out by means of enzyme immunoassay (EIA).

Results of the detection of yeast-derived components in a sample obtained after the salting-out step are shown in Table 4. The sample was subjected to the measurement after adjusting the albumin concentration to 250 mg/ml.

Total content of the yeast-derived proteins in the 250 mg/ml albumin solution was found to be 1,360 ng/ml in the case of a sample obtained after treatment by the hydrophobic chromatography and was 5.4 ng/ml in an anion exchanger-treated sample, thus showing reduction of the total content to a level of 1/250 by the latter purification step. In addition, the yeast-derived proteins were not detected in the purified sample obtained after the salting-out step at a detection limit.

[viii] Properties of the Purified HSA

(1) Molecular weight

Measurement of molecular weight was carried out in accordance with the aforementioned HPLC gel filtration procedure. Molecular weight of the purified HSA of the present invention was found to be about 67,000, which was almost the same as that of the plasma-derived HSA.

(2) Isoelectric point

Isoelectric point was measured by polyacrylamide gel electrophoresis, for example, using phastsystem ® (Pharmacia). Isoelectric point of the purified HSA of the present invention was found to be about 4.9, which was almost the same as that of plasma-derived HSA.

(3) Coloring degree

Absorbances at 280 nm, 350 nm and 450 nm were measured, and coloring degrees were calculated as an A_{350}/A_{280} ratio and an A_{450}/A_{280} ratio. The A_{350}/A_{280} ratio and the A_{450}/A_{280} ratio of the purified HSA of the instant invention were found to be about 0.02 and about 0.01, respectively, which were almost the same as those of plasma-derived HSA.

EXAMPLE 2

The culture broth obtained in Reference Example 2 was treated in the same manner as the procedures described in Example 1. Properties of the thus purified HSA were almost the same as those of the purified HSA disclosed in Example 1, in terms of molecular weight, isoelectric point and coloring degree, as well as polysaccharide content, gel filtration pattern and content of yeast-derived components.

EXAMPLE 3

A 1 ml portion of the 25% solution of purified HSA obtained in Example 1 was mixed with 1 g of DIAION CRB02 ® (a chelate resin having a styrene-divinylbenzene copolymer as the carrier portion and an N-methylglucamine group as the ligand portion, manufactured by

Mitsubishi Kasei Corp.), and the resulting mixture was stirred for 24 hours at room temperature at pH 6.8 and ionic strength of 5 mmho. The resin then was washed with distilled water to recover the non-absorbed fraction. Thereafter, the thus purified HSA was assessed for the properties as set forth in Example 1. Molecular weight, isoelectric point and gel filtration pattern of the thus obtained HSA were the same as those of the HSA obtained in Example 1.

(1) Analysis of yeast-derived components

Results of the detection of yeast-derived components in the 25% HSA solution by EIA, together with the results of Example 1, are shown in Table 4.

TABLE 4

Results of the detection of yeast-derived components

Sample	Protein content (HSA purity)	Polysaccharide content (HSA purity)
Anion exchanger treatment (steps (i) to (iv) of Ex. 1)	5.4 ng/ml (99.99999784%)	40 ng/ml
Salting-out treatment (steps (i) to (v) of Ex. 1)	<0.1 ng/ml (99.99999996%)	4 ng/ml (99.9999984%)
Steps (i) to (iv) of Ex. 1 and chelate resin treatment	Not measured	4 ng/ml (99.9999984%)
Steps (i) to (v) of Ex. 1 and chelete resin treatment	<0.1 ng/ml (99.99999996%)	<1 ng/ml (99.9999996%)

(2) Coloring degree

Absorbances at 280 nm, 350 nm, 450 nm and 500 nm were measured, and coloring degrees were calculated as an A_{350}/A_{280} ratio, an A_{450}/A_{280} ratio and an A_{500}/A_{280} ratio. The A_{350}/A_{280} ratio, the A_{450}/A_{280} ratio and the A_{500}/A_{280} ratio of the HSA of the instant invention were found to be about 0.02, about 0.01 and about 0.002, respectively, which were almost the same as those of plasma-derived HSA.

(3) Linked fatty acid content

Measurement was carried out using NFEA-Test Wako® (Wako Pure Chemical Industries, Ltd.). The linked fatty acid content was about 1.6 moles (per mole of HSA) before the chelate resin treatment but was sharply reduced by that treatment to 0.037 mole per mole of HSA.

EXAMPLE 4

The culture broth obtained in Reference Example 2 was treated in the same manner as in Example 1. Properties of the 25% solution of thus purified HSA were almost the same as those disclosed in Examples 1 and 3, in terms of molecular weight, isoelectric point, coloring degree, gel filtration pattern and content of yeast-derived components.

EXAMPLE 5

The 25% solution of the HSA obtained in Example 3 was checked for absorbances at 280 nm and 500 nm. In that case, the coloring degree was calculated as an A_{500}/A_{280} ratio. In addition, other HSA samples were prepared by repeating the process of Example 3, except that DIAION CR20® (Mitsubishi Kasei Corp.) or LEWATIT TP214® (Bayer) was used instead of DIAION CRB02®, and the absorbances were measured to calculate coloring degrees. The results are shown in Table 5. As controls, several HSA samples were prepared using carriers other than the chelate resin of the present invention, as well as other types of cation exchanger, anion exchanger and hydrophobic chromatography carrier, with the results also shown in Table 5.

TABLE 5

Carrier	Ligand	Coloring degree Resin Trade name	Coloring*
Invention			
Styrene-divinyl benzene copolymer	N-methylglucamine group	DIAION CRBO2® (Mitsubishi)	0.2
Styrene-divinyl benzene copolymer	$-NH(CH_2CH_2NH)_nH$	DIAION CR20® (Mitsubishi)	0.2
Styrene-divinyl benzene copolymer	$-NHCSNH_2$	LEWATIT TP214® (Bayer)	0.2
Control			
Styrene-divinyl benzene copolymer	DEAE	DEAE-Toyopearl® (Tosoh)	0.5
Agarose	iminodiacetic acid	Chelating-Sepharose® (Pharmacia)	0.8
Anion exchanger		DEAE Sepharose® (Pharmacia)	0.6
Hydrophobic chromatography		Phenyl Cellulofine® (Pharmacia)	0.6

Note (*): Coloring degree in an OD_{500}/OD_{280} ratio which was defined as 1.0 in the case of the purified HSA obtained through Reference Example 1 and Example 1.

EXAMPLE 6

Measuring Wave Length

Absorbance of the 25% solution of HSA obtained in Example 3 was measured at wave lengths of from 350 nm to 650 nm. Decreasing degrees of absorbance were found. The lowest ratio was found at a wave length of 500 nm. The results are shown in Table 6.

TABLE 6

	Decreasing degree of absorbance Decreasing ratio (%)
A_{350}/A_{280}	57
A_{400}/A_{280}	37
A_{450}/A_{280}	25
A_{500}/A_{280}	20
A_{550}/A_{280}	34
A_{600}/A_{280}	54
A_{650}/A_{280}	56
	Starting material: 100%

EXAMPLE 7

The process of Example 3 was repeated except that DIAION CR20® (Mitsubishi Kasei Corp.) was used instead of the chelate resin DIAION CRB02 ®in the treatment step using 1 ml of the 25% recombinant HSA solution. The DIAION CR20 ®is a chelate resin having a $—NH(CH_2CH_2NH)_nH$ group as the ligand attached to a styrene-divinylbenzene copolymer carrier. When the thus recovered HSA was checked for linked fatty acids, similar results to those of the case of Example 6 were obtained.

EXAMPLE 8

The process of Example 3 was repeated except that LEWATIT TP214 ® (Bayer) was used instead of the chelate resin DIAION CRB02 ®in the treatment step of 1 ml of the 25% recombinant HSA solution. LEWATIT TP214 ®is a chelate resin having a $—NHCSNH_2$ group as a ligand attached to the styrene-divinylbenzene copolymer carrier. When the thus recovered HSA was checked for linked fatty acids, similar results to those of the case of Example 6 were obtained.

EXAMPLE 9

Purity

Antibodies against the yeast components were prepared to detect impurities of yeast components at a high level of sensitivity. After culturing non-albumin producing yeast, yeast components were partially purified from the culture supernatant and separated into a protein fraction and a polysaccharide fraction. Each fraction was used to immunize rabbits to obtain an antibody against the protein fraction and an antibody against the polysaccharide fraction. An EIA was developed using these antibodies. In EIA system, the detection limit of proteins and polysaccharides were 0.1 ng/ml and 1 ng/ml, respectively. In purified recombinant HSA obtained in Example 3, at a concentration of 250 mg/ml, no yeast components were detected using this EIA system. That is, the contents of yeast-derived proteins and polysaccharides in purified recombinant albumin at a concentration of 25% were less than 0.1 ng/ml and less that 1 ng/ml, respectively. In other words, the purity of recombinant albumin was greater than 99.999999%. Contaminated DNA was assayed by the threshold method as described in Science, 240, 1182 (1988). In purified recombinant albumin, no DNA was detected in a 2 ml extract of a 25% albumin solution. Because the detection limit of this system was 4 pg/2 ml of 25% albumin, the amount of the contaminating DNA was less than that value. Pyrogens were measured using a reagent kit, Endospacy (Seikagaku Corp.). The pyrogen content was less than 0.1 EU/ml of 25% recombinant albumin, a sufficiently low level. In the pyrogen test using rabbits, there was no temperature rise up to a dosage of 2.5 grams per kilogram.

Composition and Structure

To ascertain the composition and structure of recombinant albumin, amino acid analysis was conducted and the CD spectrum was measured. The amino acid composition and N and C terminal sequence of recombinant albumin were identical to those of plasma albumin. Those results were consistent with the sequence of the c-DNA. Peptide mapping of recombinant albumin was carried out. Albumin was degradated by lysylendopeptiase, then each peptide was separated by reverse-phase HPLC. As a result of comparing the peptide mapping patterns, the elution profile of recombinant albumin was consistent with that of plasma albumin.

To examine the higher structure of recombinant albumin, the CD spectrum of albumin was measured. The CD spectrum of recombinant albumin was identical in the shape and magnitude to that of plasma albumin in the region of 350 nm to 195 nm.

Biological Characterization

One of the most important biological functions of albumin is ligand binding. Albumin binds various materials. The binding abilities of albumin to three typical materials were examined. Bilirubin was selected to represent pigment. Warfarin was used to represent drugs and lauric acid to represent to fatty acids. The binding of those three materials was analyzed using a Scatchard plot model. Binding constants and the number of binding sites of those three materials to recombinant albumin were consistent with those of plasma albumin. Binding curves of lauric acid to albumin are shown in FIG. 2 as an example.

As shown in FIG. 2, binding curve of recombinant albumin was consistent with plasma albumin.

The results that binding affinities of recombinant albumin with those ligands were almost similar to those of plasma albumin indicate the biological equivalency between both albumins.

Pre-Clinical Study

Preliminary data was collected during a pre-clinical study conducted in animals. The half life of recombinant albumin in dog blood was almost identical to that of plasma albumin (recombinant albumin; 6.3±0.5 day, plasma albumin; 6.0±0.7 day).

The following hemodynamic parameters were tested in dogs: blood pressure; central venous pressure; pulmonary artery pressure; cardiac output; blood gas; respiration; and electrocardiogram. At doses of 0.5 and 1.5 g/kg, the same results were obtained for recombinant albumin as for plasma albumin.

The results of a pyrogen test using rabbits showed no temperature rise up to a dose of 2.5 g/kg.

An acute toxicity test of recombinant albumin demonstrated no toxicity in monkeys and rats up to a dosage of 12.5 g/kg.

EXAMPLE 10

In an appropriate volume of distilled water for injection were dissolved 5 g of the yeast-derived HSA obtained in Example 1, 107.3 mg of acetyltryptophan sodium salt and 66.5 mg of sodium caprylate to obtain 20 ml of a pharmaceutical preparation containing 25% HSA. The resulting pharmaceutical preparation consisted of 25% HSA, 0.02M acetyltryptophan sodium salt and 0.02M sodium caprylate. The sodium chloride content was found to be 3.7 mg/ml, the pH was 7.0 and the osmotic pressure was about 1, as a ratio against physiologic saline.

EXAMPLE 11

Preservation stability of the HSA-containing pharmaceutical preparation obtained in Example 10 was examined. Determination of dimers (molecular weight distribution) was carried out by gel filtration analysis, coloring degree by absorbance analysis (A_{350}/A_{280}) and polymers or decomposed products by electrophoresis (SDS-PAGE). The results are shown in Tables 7 and 8. According to the instant invention, gelation of HSA did not occur. The content of dimers increased gradually

when maintained at 40° C., but polymers or decomposed products were not found.

TABLE 7

Cryopreservation test at −20° C. for 8 weeks					
Sodium acetyl tryptophan (M)	Sodium caprylate (M)	NaCl (mg/ml)	Before preservation	After preservation	Increasing ratio (fold)
(Coloring degree, A_{350}/A_{280})					
0	0	0	0.0236	0.0277	1.17
0.02	0.02	3.7	0.0250	0.0261	1.04
(Dimer content, %)					
0	0	0	0.021	0.036	
0.02	0.02	3.7	0.021	0.020	

TABLE 8

Accelerated preservation test at 40° C. for 3 months (Dimer content, %)				
Sodium acetyl tryptophan (M)	Sodium caprylate (M)	NaCl (mg/ml)	Before preservation	After preservation
0	0	0	0.018	0.052 or more
0.02	0.02	3.7	0.018	0.036

While the instant invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

What is claimed is:

1. A process for producing a recombinant human serum albumin comprising the steps of:
(1) treating a culture supernatant of a host which expresses human serum albumin, with a first ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 and then with a second ultrafiltration membrane having a molecular weight exclusive limit of from 1,000 to 50,000 to yield a first filtrate;
(2) heat-treating the first filtrate at 50° to 70° C. for 30 minutes to 5 hours to yield a heated sample;
(3) acid-treating the heated sample at a pH of from 3 to 5 to yield an acid-treated sample;
(4) treating the acid-treated sample using an ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 to yield a second filtrate;
(5) exposing the second filtrate to a cation exchanger at a pH of 3 to 5 and a salt concentration of 0.01 to 0.2M, and then exposing said cation exchanger to a pH of 8 to 10 and a salt concentration of 0.2 to 0.5M to yield a first eluate;
(6) allowing the first eluate to contact with a carrier for hydrophobic chromatography at a pH of 6 to 8 and a salt concentration of 0.01 to 0.5M, and recovering non-adsorbed fractions to yield a second eluate; and
(7) allowing the second eluate to contact with an anion exchanger at a pH of 6 to 8 and a salt concentration of 0.01 to 0.1M, and recovering non-adsorbed fractions to yield said albumin.

2. The process for producing human serum albumin according to claim 1 further comprising a salt precipitation step following step (5), step (6) or step (7), said salt precipitation step comprising exposing said first eluate, said second eluate or said albumin to a pH of 3 to 5 and a salt concentration of 0.1 to 3M to yield a precipitate and dissolving said precipitate in a buffer.

3. The process for producing human serum albumin according to claim 1 further comprising exposing said albumin of step (7) to a chelate resin and recovering non-adsorbed fractions to yield albumin.

4. The process for producing human serum albumin according to claim 3, wherein said chelate resin has an exchange group as a ligand which is capable of undergoing chelate formation selected from the group consisting of a polyol group, a polyamine group and a thiourea group.

5. The process for producing human serum albumin according to claim 3, wherein said albumin of step (7) is exposed to the chelate resin at a pH 3 to 9 and at an ionic strength of 50 mmho or less.

6. The process for producing human serum albumin according to claim 1, wherein said heat-treating step (2) is carried out in the presence of a compound selected from the group consisting of acetyltryptophan, an organic carboxylic acid and organic carboxylic acid salt.

7. The process for producing human serum albumin according to claim 1, wherein said heat-treating step (2) is carried out in the presence of a thiol compound.

8. The process for producing human serum albumin according to claim 1, wherein said heat-treating step (2) is carried out in the presence of aminoguanidine.

9. A process for producing a recombinant human serum albumin comprising the steps of:
(1) treating a culture supernatant of a host which expresses human serum albumin, with a first ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 and then with a second ultrafiltration membrane having a molecular weight exclusive limit of from 1,000 to 50,000 to yield a first filtrate;
(2) heat-treating the first filtrate at 50° to 70° C. for 30 minutes to 5 hours to yield a heated sample;
(3) acid-treating the heated sample at a pH of from 3 to 5 to yield an acid-treated sample;
(4) treating the acid-treated sample using an ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 to yield a second filtrate;
(5) exposing the second filtrate to a cation exchanger at a pH of 3 to 5 and a salt concentration of 0.01 to 0.2M, and then exposing said cation exchanger to a pH of 8 to 10 and a salt concentration of 0.2 to 0.5M to yield a first eluate;
(6) allowing the first eluate to contact with a carrier for hydrophobic chromatography at a pH of 6 to 8 and a salt concentration of 1 to 3M, and then exposing said carrier to a salt concentration of 0.01 to 0.5M, and recovering non-adsorbed fractions to yield a second eluate; and
(7) allowing the second eluate to contact with an anion exchanger at a pH of 6 to 8 and a salt concentration of 0.01 to 0.1M, and recovering non-adsorbed fractions to yield said albumin.

10. A process for producing a recombinant human serum albumin comprising the steps of:
(1) treating a culture supernatant of a host which expresses human serum albumin, with a first ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 and then with a second ultrafiltration membrane having a molecular weight exclusive limit of from 1,000 to 50,000 to yield a first filtrate;
(2) heat-treating the first filtrate at 50° to 70° C. for 30 minutes to 5 hours to yield a heated sample;

(3) acid-treating the heated sample at a pH of from 3 to 5 to yield an acid-treated sample;

(4) treating the acid-treated sample using an ultrafiltration membrane having a molecular weight exclusive limit of from 100,000 to 500,000 to yield a second filtrate;

(5) exposing the second filtrate to a cation exchanger at a pH of to 5 and a salt concentration of 0.01 to 0.2M, and then exposing said cation exchanger to a pH of 8 to 10 and a salt concentration of 0.2 to 0.5M to yield a first eluate;

(6) allowing the first eluate to contact with a carrier for hydrophobic chromatography at a pH of 6 to 8 and a salt concentration of 0.01 to 0.5M, and recovering non-adsorbed fractions to yield a second eluate; and

(7) allowing the second eluate to contact with an anion exchanger at a pH of 6 to 8 and a salt concentration of 0.001 to 0.05M, and then exposing the anion exchanger to a pH 6 to 8 and a salt concentration of 0.05 to 0.1M, and recovering non-adsorbed fractions to yield said albumin.

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A Versatile Set of Vectors for Constitutive and Regulated Gene Expression in *Pichia pastoris*

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The budding yeast *Pichia pastoris* is an attractive system for exploring certain questions in cell biology, but experimental use of this organism has been limited by a lack of convenient expression vectors. Here we describe a set of compact vectors that should allow for the expression of a wide range of endogenous or foreign genes in *P. pastoris*. A gene of interest is inserted into a modified pUC19 polylinker; targeted integration into the genome then results in stable and uniform expression of this gene. The utility of these vectors was illustrated by expressing the bacterial β-glucuronidase (GUS) gene. Constitutive GUS expression was obtained with the strong *GAP* promoter or the moderate *YPT1* promoter. The regulatable *AOX1* promoter yielded very strong GUS expression in methanol-grown cells, negligible expression in glucose-grown cells, and intermediate expression in mannitol-grown cells. GenBank Accession Numbers are: pIB1, AF027958; pIB2, AF027959; pIB3, AF027960; pIB4, AF027961. © 1998 John Wiley & Sons, Ltd.

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KEY WORDS — *Pichia pastoris*; expression vectors; gene regulation

INTRODUCTION

During the past decade, *Pichia pastoris* has been exploited by biotechnologists for the high-level production of foreign proteins (Romanos *et al.*, 1992; Cregg *et al.*, 1993). *P. pastoris* can utilize methanol as a carbon source, and growth on methanol results in strong induction of the peroxisomal enzyme alcohol oxidase (Gleeson and Sudbery, 1988). The major alcohol oxidase isozyme is encoded by the *AOX1* gene (Cregg *et al.*, 1989). Vectors containing the *AOX1* promoter have been widely used for the regulated overproduction of cytosolic and secreted proteins (Romanos *et al.*, 1992; Cregg *et al.*, 1993; Scorer *et al.*, 1994). Recently, the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter has also been employed for the strong constitutive expression of foreign genes (Waterham *et al.*, 1997).

As an experimental organism, *P. pastoris* shares many of the advantages of *Saccharomyces cerevisiae*, including mating and sporulation, transformation with integrating or replicating vectors, and gene replacement by homologous recombination (Cregg *et al.*, 1985; Cregg and Madden, 1987; Gleeson and Sudbery, 1988; Gould *et al.*, 1992; Crane and Gould, 1994). Cell biologists initially became interested in *P. pastoris* because it provides a convenient system for studying peroxisome biogenesis (Gould *et al.*, 1992; Liu *et al.*, 1992). The recent major advances in this field have

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derived largely from genetic studies of *P. pastoris* and other budding yeasts (Erdmann *et al.*, 1997). *P. pastoris* is also being used for a genetic analysis of autophagy (Tuttle and Dunn, 1995). We have chosen *P. pastoris* as a model system to study the organization of the transitional endoplasmic reticulum and Golgi apparatus, because unlike *S. cerevisiae, P. pastoris* has coherent Golgi stacks located next to discrete sites of transitional ER (Gould *et al.*, 1992; Glick, 1996; O.W.R and B.S.G., in preparation).

An essential tool for yeast cell biology is vectors for the expression of endogenous or foreign genes (Schneider and Guarente, 1991). Expression vectors have been developed for *P. pastoris* (Cregg *et al.*, 1993; Scorer *et al.*, 1994; Waterham *et al.*, 1997), but many of them are cumbersome to use because of large size and/or a limited number of restriction sites for subcloning. Here we describe a set of compact expression vectors that contain a pUC19-derived polylinker for the insertion of cloned genes. Because *P. pastoris CEN* sequences are not yet available for generating stable episomal plasmids, we created vectors that can be integrated into the chromosomal *HIS4* locus (Romanos *et al.*, 1992). The targeted integration of these vectors is very efficient, and correct integrants can be identified by a simple polymerase chain reaction (PCR) assay.

Different vectors were designed to allow for either constitutive or regulated gene expression. Strong constitutive expression can be obtained with the *GAP* promoter, and moderate constitutive expression with the promoter from the *YPT1* gene, which encodes a small GTPase involved in secretion (Segev *et al.*, 1988). Regulated expression can be obtained with the *AOX1* promoter. In the past, use of the *AOX1* promoter has yielded expression levels that were either very high (on methanol) or very low (on glucose or other carbon sources) (Tschopp *et al.*, 1987; Waterham *et al.*, 1997). However, we have found that growing cells on mannitol results in an intermediate level of expression from the *AOX1* promoter. Together, these expression vectors provide a versatile tool for the experimental manipulation of *P. pastoris*.

MATERIALS AND METHODS

General methods

Standard procedures were used for recombinant DNA manipulations (Ausubel *et al.*, 1995). The PCR was carried out with a Perkin-Elmer 2400 PCR machine using either *Pfu* or *Taq2000* DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Automated fluorescent DNA sequencing was performed by the dye terminator method using an Applied Biosystems Prism 377 machine. Unless otherwise indicated, all chemicals were purchased from Sigma (St Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Yeast growth media were as follows. YPD: 1% yeast extract, 2% peptone, 2% glucose, 20 mg/l adenine sulfate, 20 mg/l uracil. SD – His: 0·67% yeast nitrogen base, 2% glucose, complete supplement mixture minus histidine (CSM – His; Bio 101, Vista, CA). SYD: 0·67% yeast nitrogen base, 0·05% yeast extract, 0·4 mg/l biotin, 40 mg/l arginine hydrochloride, 2% glucose. SYG, SYM and SYN were similar to SYD except that instead of glucose they contained 1% glycerol, 0·5% methanol or 2% mannitol, respectively.

Plasmid construction

A modified version of the *P. pastoris HIS4* gene (Cregg *et al.*, 1985; Crane and Gould, 1994) was created as follows. pYM22, a kind gift of Jim Cregg, contained a *HIS4* gene in which the internal *Bam*HI site had been removed (Cregg and Madden, 1987). This mutant gene was subcloned as an *Eco*RI-*Bam*HI fragment into pUC19 (Yanisch-Perron *et al.*, 1985) to yield pUC19-HIS4. Next, a 612-bp internal fragment of *HIS4* was amplified by PCR from pYM22 with *Pfu* polymerase using the primers GCACCCTTACC CCAGAAGTCATCT and GTGCCGGAGGAG TAATCTCCACAA. The amplified fragment was cloned into pUC19-HIS4 that had been digested with *Kpn*I and blunt-ended with T4 DNA polymerase. The resulting plasmid, pUC19-HIS4*, contains two point mutations that eliminate the *Kpn*I sites in *HIS4* without altering the corresponding amino acids.

pIB1 was constructed as follows. First, the complementary oligonucleotides GATCCACTA GTCTCGAGCTGCA and GCTCGAGACTAG TG were annealed, and the resulting double-stranded fragment was inserted into pUC19 that had been digested with *Bam*HI and *Pst*I. In the resulting plasmid, termed pUC19-SX, the *Xba*I and *Sal*I sites in the polylinker were replaced with *Spe*I and *Xho*I sites, respectively. Next, a 260-bp fragment containing the transcription terminator

of *P. pastoris AOX1* (Koutz *et al.*, 1989) was amplified by PCR from pPIC3K (Scorer *et al.*, 1994) with *Pfu* polymerase using the primers GCCGTCGCCAAGCTTCTTAGACATGACTG TTCCTCAGTTC and GGGACATGTGTGGG AAATACCAAGAAAAACATC. The PCR product was digested with *Hin*dIII and *Afl*III and inserted into pUC19-SX that had been cut with the same enzymes, yielding pUC19-SX-AOXTT. Finally, the modified *HIS4* gene from pUC19-HIS4* was excised with *Bgl*II, blunt-ended with Klenow enzyme, and subcloned into pUC19-SX-AOXTT that had been digested with *Ssp*I, yielding pIB1. Sequencing confirmed that no undesired mutations had been introduced into the polylinker or *AOX1* transcription terminator portions of pIB1.

pIB2 was constructed as follows. The promoter of the *P. pastoris GAP* gene was excised from pHWO10 (Waterham *et al.*, 1997) by digesting with *Bst*YI and blunt-ending with Klenow enzyme, then digesting with *Eco*RI. This fragment was subcloned into pIB1 that had been digested with *Nde*I, blunt-ended with Klenow enzyme, and digested with *Eco*RI.

pIB3 was constructed as follows. A 504-bp fragment containing the promoter of the *P. pastoris YPT1* gene (I.B.S. and B.S.G., unpublished data) was amplified by PCR from plasmid DNA with *Pfu* polymerase using the primers CGAG GCATACATATGATGAGTCACAATCTGCTT CCA and GTGAATTCGACTGCTATTATCT CTGTGTGTA. The PCR product was digested with *Nde*I and *Eco*RI and cloned into pIB1 that had been digested with the same enzymes.

pIB4 was constructed as follows. The promoter of the *P. pastoris AOX1* gene was excised from pHIL-D2 (Despreaux and Manning, 1993) by digesting with *Sac*I and blunt-ending with T4 DNA polymerase, then digesting with *Eco*RI. This fragment was subcloned into pIB1 that had been digested with *Nde*I, blunt-ended with Klenow enzyme, and digested with *Eco*RI.

The β-glucuronidase (GUS) gene was excised from pBI101 (Clontech, Palo Alto, CA) by digesting with *Sac*I, blunt-ending with T4 DNA polymerase, and digesting with *Bam*HI. This fragment was subcloned into pIB2, pIB3, and pIB4 after digestion with *Xho*I, blunt-ending with Klenow enzyme, and digestion with *Bam*HI. The resulting constructs (pIB2-GUS, pIB3-GUS and pIB4-GUS) were linearized with *Sal*I and integrated into the *HIS4* locus as described below.

Transformation of P. pastoris *by electroporation*

This procedure was adapted from the method of Becker and Guarente (1991). A 50-ml culture of PPY12, a *his4 arg4* auxotrophic strain of *P. pastoris* (Gould *et al.*, 1992), was grown in YPD at 30°C with good aeration to an OD_{600} of 1–2. The culture was then supplemented with 1 ml of 1·0 M-Na^+-HEPES, pH 8·0 and 1 ml of 1·0 M-DTT, and incubation was continued for an additional 15 min at 30°C. The cells were transferred to a chilled 50-ml Falcon tube, centrifuged for 3 min at 2000 *g* (3000 rpm) in a tabletop centrifuge at 4°C, and resuspended in 50 ml ice-cold double-distilled H_2O. This H_2O wash step was repeated, followed by a wash with 20 ml cold 1·0 M-sorbitol. Finally, the cells were resuspended in 200 µl cold 1·0 M-sorbitol. A 40-µl aliquot of yeast cells was mixed with 0·1–1 µg DNA in ≤5 µl of a low-salt solution. This mixture was transferred to a chilled electroporation cuvette (0·2 cm gap) and pulsed with a Bio-Rad Gene Pulser set at 1·5 kV, 25 µF, 200 ohms (time constant ~5 ms). The cell suspension was immediately diluted with 1 ml of cold 1·0 M-sorbitol and transferred to a 1·5-ml centrifuge tube. After centrifugation for 1 min at 2000 *g* (5000 rpm) in a microfuge at room temperature, the upper 800 µl of liquid was removed and the cells were gently resuspended in the remaining volume. Finally, the cells were spread on SD − His plates supplemented with 1·0 M-sorbitol, and the plates were incubated at 30°C for 2–3 days until colonies appeared.

Integration of pIB vector constructs into the HIS4 *locus*

Two µg of a pIB vector (with or without an insert in the polylinker) was digested with either *Sal*I or *Stu*I, ethanol-precipitated, washed twice with cold 70% ethanol, and resuspended in 10 µl H_2O. For transformation, 2·5 µl (0·5 µg) of linearized DNA was used in an electroporation. Six separate transformants were colony-purified by restreaking on SD − His plates. Genomic DNA was then isolated from each transformant using a small-scale Easy DNA preparation (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For each 50-µl PCR reaction, 0·25 µl (~0·5 µg) of genomic DNA was added to a mixture containing PCR buffer, 200 µM of each dNTP, 0·5 µM each of the primers GCTGTT AAGGTTCGTATGGAGAAAC (sense) and GT GTAGTCTTGAGAAATTCTGAAG (antisense),

and 0·25 µl of *Taq2000* DNA polymerase that had been preincubated with 0·25 µl of TaqStart antibody (Clontech, Palo Alto, CA). The PCR mixtures were incubated for 2 min at 94°C, followed by 30 cycles of: denaturation, 10 s at 94°C; annealing, 30 s at 55°C; elongation at 68°C. The elongation time was 40 s per kilobase of expected amplified product, with an increment of 20 s per cycle during cycles 11–30. The reactions were terminated by a 7-min incubation at 68°C followed by a hold of 4°C. Twenty µl of each reaction mixture was analysed on an agarose gel. As a positive control, a parallel PCR amplification was performed using 10 ng of the starting pIB vector in place of genomic DNA.

Measurement of promoter activities using GUS as a reporter gene

Yeast strains transformed with pIB2, pIB2-GUS, pIB3-GUS or pIB4-GUS were grown as precultures in 5 ml of YPD in a 15-ml Falcon tube for 24 h at 30°C. These precultures could be stored at 4°C for up to a month. Aliquots of the precultures were diluted into 50 ml of medium using the following dilutions: SYD, 1:50,000; SYG, 1:15,000; SYM, 1:1000; SYN, 1:1000. These cultures were grown at 30°C with good aeration for approximately 20 h to an OD_{600} of 0·4–0·6. Cells were harvested by centrifugation for 3 min at 1000 *g* (2000 rpm) in a tabletop centrifuge and resuspended in 2 ml of freshly-made extraction buffer: 50 mM-sodium phosphate, pH 7·0, 10 mM-β-mercaptoethanol, 5 mM-EDTA, 0·1% Triton X-100, 0·25 mM-phenylmethylsulfonyl fluoride, 1 µM-pepstatin. The suspension was transferred to a 15-ml Falcon tube and cooled on ice. Acid-washed glass beads (0·5 mm diameter) were added to give a total volume of 3 ml. The mixture was vortexed for 30 s at top speed, then cooled on ice for 30 s. This procedure was repeated five times in total, followed by centrifugation for 3 min at 1000 *g* at 4°C. The supernatant was then centrifuged for 15 min at 100,000 *g* (50,000 rpm) in a Beckman Optima TLX ultracentrifuge at 4°C. The clarified supernatant was divided into 200-µl aliquots, which were frozen in liquid N_2 and stored at −80°C. One aliquot was used to measure the protein concentration using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard; protein concentrations in the cell extracts ranged from 0·3 to 1·1 mg/ml.

For GUS assays, cell extracts were either left undiluted or else diluted 5- to 300-fold in extraction buffer, depending upon the amount of GUS present. Enzyme activity was measured by a fluorometric assay (Gallagher, 1992). A substrate solution of 2·5 mM-4-methylumbelliferyl β-D-glucuronide was prepared in extraction buffer lacking protease inhibitors. Forty µl of diluted or undiluted extract was added to 160 µl of substrate solution, and the tube was transferred to a 37°C water bath. After 5, 10 and 15 min, 50-µl aliquots were transferred to tubes containing 2 ml 0·2 M-Na_2CO_3 (stop buffer). A zero-minute time point was obtained by adding 10 µl of extract to a tube containing 2 ml of stop buffer and 40 µl of substrate solution. Fluorescence was measured on a Perkin-Elmer LS 50 luminescence spectrometer using an excitation wavelength of 365 nm, an emission wavelength of 460 nm and a slit width of 5·0 nm. The fluorescence values were translated into pmol of 4-methylumbelliferone product based on the signal obtained with known amounts of this compound. Enzymatic activity was then calculated with the KaleidaGraph program (Synergy Software, Reading, PA) by fitting the time course results to a straight line and determining the slope.

RESULTS AND DISCUSSION

Features of the pIB vectors

Figure 1 shows a diagram of pIB1. This plasmid was derived from the common cloning vector pUC19 by (a) inserting a modified *P. pastoris HIS4* gene, and (b) placing the transcription terminator region from *AOX1* immediately downstream of the polylinker. Because *HIS4* contains both *Xba*I and *Sal*I sites, these two sites in the polylinker were replaced with compatible *Spe*I and *Xho*I sites, respectively. pIB1 is a useful vector for expressing genes containing endogenous promoters that function in *P. pastoris*.

The expression vectors pIB2, pIB3 and pIB4 were derived from pIB1 by placing the promoters from the *P. pastoris* genes for glyceraldehyde-3-phosphate dehydrogenase (*GAP*; Waterham *et al.*, 1997), *YPT1* (I.B.S. and B.S.G., unpublished data) or alcohol oxidase (*AOX1*; Koutz *et al.*, 1989), respectively, immediately upstream of the polylinker (Figure 1; Table 1). A gene inserted into the polylinker of one of these vectors will be expressed under control of the adjacent promoter. The presence of the *AOX1* transcription terminator ensures that the 3′-end of the transcript will be processed correctly (Koutz *et al.*, 1989).

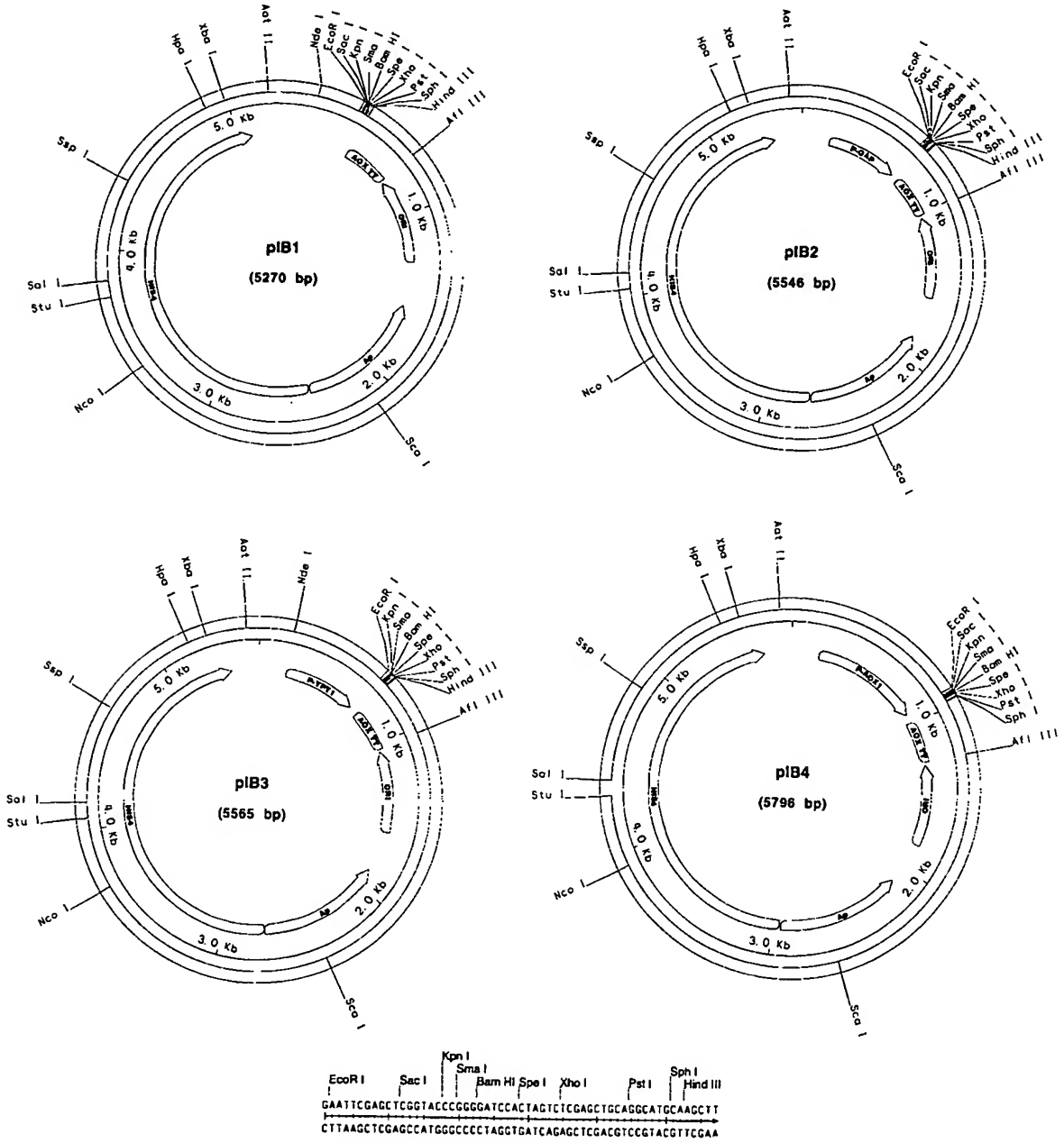

Figure 1. Structures of the pIB vectors and sequence of the polylinker. Unique restriction sites are indicated, pIB2, pIB3 and pIB4 were derived from pIB1 by inserting the appropriate promoters between the *Nde*I and *Eco*RI sites. In all cases the *Eco*RI site was preserved; the *Nde*I site was preserved in pIB3 but destroyed in pIB2 and pIB4. The *Sac*I site is no longer unique in pIB3, and the *Hin*dIII site is no longer unique in pIB4. Note that the *Sph*I recognition sequence contains an ATG triplet, so the 5'-end of an open reading frame must be inserted into pIB2, pIB3 or pIB4 upstream of the *Sph*I site. AOX TT, transcription terminator from *P. pastoris AOX1*; ORI, ColE1 origin of replication; Ap, β-lactamase gene conferring ampicillin resistance; HIS4, *P. pastoris HIS4*; P-GAP, promoter from *P. pastoris GAP*; P-YPT1, promoter from *P. pastoris YPT1*; P-AOX1, promoter from *P. pastoris AOX1*.

Table 1. Properties of the pIB vectors

Plasmid name	Size (bp)	GenBank accession no.	Promoter	Promoter characteristics
pIB1	5270	AF027958	(None)	
pIB2	5546	AF027959	*GAP*	Strong constitutive
pIB3	5565	AF027960	*YPT1*	Moderate constitutive
pIB4	5796	AF027961	*AOX1*	Very strong on methanol; strong on mannitol; weak on glycerol; off on glucose

For integration into the *P. pastoris* genome, a construct is linearized by digestion within the *HIS4* locus (Rothstein, 1991; Romanos *et al.*, 1992). The *Sal*I and *Stu*I sites are available for this purpose. (If a gene of interest contains both *Sal*I and *Stu*I sites, it should still be possible to generate a linearized plasmid by partial digestion with one of these enzymes.) The linearized plasmid is transformed into a histidine auxotroph that contains point mutations in the chromosomal *HIS4* gene. Transformants are then selected on plates lacking histidine.

Assessment of correct integration

If a linearized pIB construct integrates into the *P. pastoris* genome outside of the *HIS4* locus, the strain will remain unable to grow in the absence of histidine because the plasmid-borne *HIS4* gene has been interrupted. Only integrants that insert correctly will regenerate a functional *HIS4* gene. However, even in the absence of integration, a functional chromosomal copy of *HIS4* could be generated by a gene conversion event in which the plasmid-borne sequences act as donor (Rothstein, 1991). To determine whether a transformant has correctly integrated the pIB construct, we prepare genomic DNA and perform a PCR reation, using a sense primer that hybridizes near the 3'-end of *HIS4* and an antisense primer that hybridizes near the 5'-end of *HIS4* (see Materials and Methods). An amplified product will be obtained only if an integration event has generated two nearby copies of *HIS4*. The size of this product will be approximately 3 kb (the size of the pIB vector minus the size of *HIS4*) plus the size of the gene inserted into the polylinker. Using this method, we have readily detected integration with inserted genes as large as 6·5 kb (not shown).

In our experience using different pIB vectors and various inserted genes, we consistently find that about 50% of the transformants contain correct integrations (not shown). Thus, analysis of six independent transformants normally yields at least one positive clone.

Because integration of a pIB construct generates two copies of *HIS4*, the integrated plasmid might occasionally be excised from the chromosome via homologous recombination (Rothstein, 1991; Romanos *et al.*, 1992). In practice, however, we have not observed such instability. For example, when a strain expressing an epitope-tagged protein was examined by immunofluorescence, the cells exhibited uniform staining, even if the culture had been grown in non-selective YPD medium (not shown).

Measurement of promoter activities

The β-glucuronidase (GUS) gene from *Escherichia coli* has proven to be a useful reporter for measuring promoter activity in a variety of organisms (Gallagher, 1992), including the budding yeasts *S. cerevisae* and *Yarrowia lipolytica* (Marathe and McEwen, 1995; Bauer *et al.*, 1993). Neither of these yeasts shows significant levels of endogenous GUS activity. Similarly, we observed a very low background signal in GUS assays with extracts from pIB2-transformed *P. pastoris* cells (Table 2 legend). However, when a pIB2-GUS construct was integrated into the *P. pastoris* genome, enzyme activity was observed (Table 2). The specific activity of GUS expressed from the *GAP* promoter in *P. pastoris* was comparable to the activity reported for galactose-grown *S. cerevisiae* cells expressing GUS from the strong *GAL1* promoter (Marathe and McEwen, 1995). When GUS was expressed in *P. pastoris* from the *YPT1* promoter present in pIB3, a weaker but still significant enzyme activity was detected (Table 2). Expression levels from the *YPT1* promoter were about 10- to 100-fold lower than those from the

Table 2. Activities of the various promoters in cells grown on different carbon sources

Integrated plasmid	Carbon source	GUS activity
pIB2-GUS (*GAP* promoter)	Glucose	70·4
	Glycerol	48·8
	Methanol	11·3
	Mannitol	18·8
pIB3-GUS (*YPT1* promoter)	Glucose	0·63
	Glycerol	0·84
	Methanol	0·45
	Mannitol	1·67
pIB4-GUS (*AOX1* promoter)	Glucose	0·05
	Glycerol	0·34
	Methanol	587·6
	Mannitol	20·3

As described in Materials and Methods, extracts from cells transformed with the indicated plasmids were assayed for GUS activity, which is given in units of pmol 4-methylumbelliferone produced per min per μg cellular protein. Activities represent the means from two separate experiments. To measure background GUS activity in the cells, extracts were prepared from a strain transformed with the empty pIB2 vector. The following background values were subtracted from the numbers shown: glucose, 0·03; glycerol, 0·02; methanol, 0·08; mannitol, 0·02.

GAP promoter. Therefore, pIB3 should be useful for expressing genes that would be toxic when overexpressed.

As previously reported (Waterham *et al.*, 1997), the *GAP* promoter was constitutively active on various carbon sources (Table 2). The *YPT1* promoter was also constitutively active (Table 2), as would be expected given the important role of Ypt1p in the operation of the secretory pathway (Segev *et al.*, 1988). In contrast, expression from the *AOX1* promoter present in pIB4 was strongly regulated by the carbon source. Very high levels of GUS activity were observed in methanol-grown cells, but virtually no activity was detectable in glucose-grown cells (Table 2). These data are consistent with previous reports that the *AOX1* promoter is strongly induced by methanol and repressed by glucose (Tschopp *et al.*, 1987). In glycerol-grown cells, the *AOX1* promoter drove a low but measurable level of GUS expression (Table 2). Low-level expression from the *AOX1* promoter has also been observed on oleic acid-grown cells (Waterham *et al.*, 1997).

Although massive overexpression from the methanol-induced *AOX1* promoter is desirable for biotechnology, such strong expression is often a disadvantage for cell biology experiments. In *S. cerevisiae* the *GAL1* promoter is strongly induced by galactose and repressed by glucose, but intermediate expression levels are observed in cells grown on raffinose (Johnston *et al.*, 1994). Similarly, when *P. pastoris* cells were grown on mannitol, GUS expression from the *AOX1* promoter was approximately 30-fold lower than in methanol-grown cells (Table 2). Thus, mannitol appears to be a useful carbon source for obtaining an intermediate level of expression from the *AOX1* promoter.

It was recently reported that the *GAP* promoter is similar in strength to the methanol-induced *AOX1* promoter (Waterham *et al.*, 1997). However, we found that GUS expression from the *AOX1* promoter in methanol-grown cells was about eight-fold higher than expression from the *GAP* promoter in glucose-grown cells (Table 2). This result fits with the observation that alcohol oxidase can comprise up to 30% of the total protein in methanol-grown *P. pastoris* cells (Couderc and Baratti, 1980). The difference between our data and those from the earlier study may be due to the use of different reporter genes (GUS versus β-lactamase), or may reflect the use of a different polylinker for inserting cloned genes.

In conclusion, we have created a series of integrating vectors that allow for stable, uniform expression of cloned genes in *P. pastoris*. By choosing the appropriate promoter and carbon source, one can obtain various levels of constitutive or regulated expression. Promoter activities can be quantified using the GUS reporter gene. These techniques will facilitate the use of *P. pastoris* as a model organism for cell biology.

The set of four pIB vectors is available free of charge upon request.

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Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter

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Abstract

We report the cloning and sequence of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*) from the yeast *Pichia pastoris*. The gene is predicted to encode a 35.4-kDa protein with significant sequence similarity to glyceraldehyde-3-phosphate dehydrogenases from other organisms. Promoter studies in *P. pastoris* using bacterial β-lactamase as a reporter showed that the *GAP* promoter (P_{GAP}) is constitutively expressed, although its strength varies depending on the carbon source used for cell growth. Expression of β-lactamase under control of P_{GAP} in glucose-grown cells was significantly higher than under control of the commonly employed alcohol oxidase 1 promoter (P_{AOX1}) in methanol-grown cells. As an example of the use of P_{GAP}, we showed that β-lactamase synthesized under transcriptional control of P_{GAP} is correctly targeted to peroxisomes by addition of either a carboxy-terminal or an amino-terminal peroxisomal targeting signal. P_{GAP} has been successfully utilized for synthesis of heterologous proteins from bacterial, yeast, insect and mammalian origins, and therefore is an attractive alternative to P_{AOX1} in *P. pastoris*.

Keywords: Heterologous gene expression; Expression vector; β-Lactamase reporter; Peroxisomal protein import; Yeast

1. Introduction

Pichia pastoris is a budding yeast exploited for academic and commercial purposes. The yeast is a commonly employed host system for the production of heterologous proteins (Cregg et al., 1993). In this system, foreign genes are typically expressed under control of the *P. pastoris* alcohol oxidase 1 promoter (P_{AOX1}). P_{AOX1} is tightly regulated by a carbon source-dependent repression/induction mechanism; i.e., its expression is fully repressed during growth of the yeast on glucose or glycerol, and maximally induced during growth on methanol (Tschopp et al., 1987). A major advantage of this tight regulation is that foreign genes whose products are toxic to the cell can be readily introduced and maintained in *P. pastoris* by culturing the yeast under repressing growth conditions to prevent selection of nonexpressing strains. However, for production of certain heterologous proteins the use of methanol for induction may be inappropriate or inconvenient. In such cases, a strong constitutive promoter may be a practical alternative.

For academic research, *P. pastoris* has evolved as a major model organism to study the molecular mechanisms involved in peroxisome biogenesis (Subramani,

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Abbreviations: *amp*, *E. coli* gene encoding β-lactamase; AOX, alcohol oxidase; *AOX1*, *P. pastoris* gene encoding alcohol oxidase 1; *bla*, modified *amp* gene; bp, base pair(s); *CPT*, mammalian cDNA encoding a carnitine palmitoyltransferase; *E.*, *Escherichia*; *GAP*, *P. pastoris* gene encoding glyceraldehyde-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *gMDH*, watermelon cDNA encoding glyoxysomal malate dehydrogenase; *H.*, *Hansenula*; *HIS4*, *P. pastoris* gene encoding histidinol dehydrogenase; kb, kilobase(s) or 1000 bp; kDa, kilodalton; NAD, nicotinamide-adenine dinucleotide; OD, optical density; *P.*, *Pichia*; p, plasmid; *P*, promoter; *PAF1*, mammalian cDNA encoding peroxisome assembly factor-1; *PER*, peroxisome biogenesis gene; PTS, peroxisomal targeting signal; *S.*, *Saccharomyces*; *T*, terminator of transcription; *TDH*, *S. cerevisiae* gene encoding a glyceraldehyde-3-phosphate dehydrogenase.

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1993; Waterham and Cregg, 1997). Peroxisomes are ubiquitous eukaryotic organelles which in *P. pastoris* cells are massively induced when the yeast is grown on the carbon sources methanol or oleic acid (Gould et al., 1992; Liu et al., 1992). As a tool for certain experiments in our studies on peroxisome biogenesis, we needed a strong promoter which would constitutively express genes in *P. pastoris* under both peroxisome-inducing and non-inducing growth conditions.

In this paper, we report the isolation and sequence of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*). We show that its promoter, P_{GAP}, constitutively expresses genes in *P. pastoris* cells grown on glucose, glycerol, methanol, or oleic acid. In addition, we show that bacterial β-lactamase expressed under transcriptional control of P_{GAP} can be used as a suitable reporter protein to study peroxisomal protein import in *P. pastoris*.

2. Results and discussion

2.1. Isolation of the GAP gene

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a tetrameric NAD-binding enzyme involved in glycolysis and gluconeogenesis and is often expressed constitutively and at high levels. The *P. pastoris GAP* gene was isolated from a *P. pastoris* genomic DNA library (Cregg et al., 1985) by colony hybridization (Sambrook et al., 1989) using as a hybridization probe a 2.1-kb *Hin*dIII fragment containing *TDH3*, one of the three genes of *Saccharomyces cerevisiae* encoding GAPDH (Holland and Holland, 1979; Musti et al., 1983; McAllister and Holland, 1985b). Several genomic clones were isolated which had overlapping restriction patterns. The approximate location of the *GAP* gene was determined by Southern blot analysis of restriction fragments from the genomic clones using the *S. cerevisiae TDH3* gene as a hybridization probe. Selected restriction fragments were subcloned and sequenced in both directions using the dideoxy chain termination method of Sanger et al. (1977).

2.2. Sequence analysis of the GAP gene and its product

The DNA sequence of a 1.87-kb genomic fragment was determined starting from a *Bam*HI site located 493 bp upstream from the translation initiation ATG and revealed an open reading frame of 999 bases with the potential to encode a polypeptide of 333 amino acids with a calculated mass of 35.4 kDa (Fig. 1). Southern blot analysis of *P. pastoris* genomic DNA using part of this 1.87-kb fragment (not shown) or the *S. cerevisiae TDH3* gene (Fig. 2A) as a hybridization probe indicated that the *P. pastoris* genome contains only one *GAP* gene, in contrast to the *S. cerevisiae* genome which contains three genes (Fig. 2A; McAllister and Holland, 1985b).

The predicted primary sequence revealed several structural features characteristic of GAPDHs, including a NAD-binding domain (amino acid residues 1–147) and conserved amino acid residues Cys^{151}, His^{178} and Lys^{185} that are thought to be involved in catalysis (Olsen et al., 1975). The residues surrounding the active site residue Cys^{151}, namely Ala-Ser-Cys^{151}-Thr-Thr-Asn-Cys-Leu, exactly matched the consensus sequence that is conserved in both prokaryotic and eukaryotic GAPDHs.

As expected, the primary sequence showed a high degree of similarity to eukaryotic and prokaryotic GAPDHs listed in the protein databases. The highest level of similarity was observed with the *S. cerevisiae TDH1*, *TDH3* (both 80% identical) and *TDH2* genes (79% identical).

Northern blot analysis of total RNA isolated from *P. pastoris* cells grown on glucose, glycerol or methanol using a *GAP*-specific hybridization probe indicated that the *GAP* gene is constitutively transcribed on all three carbon sources, although the mRNA levels varied depending on the carbon source. Highest mRNA levels were observed in glucose-grown cells. In glycerol-grown cells, levels were approximately two-thirds and in methanol-grown cells approximately one-third of the levels observed in glucose-grown cells (Fig. 2B).

2.3. Construction of a P_{GAP}-controlled expression vector

To enable expression of genes under the transcriptional control of P_{GAP}, a 0.95-kb *Eco*RI-*Bgl*II P_{AOX1} fragment from the *P. pastoris-Escherichia coli* shuttle vector pHIL-A1 (Invitrogen, San Diego, CA, USA) was replaced with a 0.5-kb *Eco*RI-*Bam*HI P_{GAP} fragment (Fig. 3). This P_{GAP} fragment was generated by amplification via the polymerase chain reaction using as 5′-primer 5′-GCAGCGGATCCTTTTTTGTAG-3′ and as 3′-primer 5′-AAGAATTCTTGATAGTTGTTC-AATTG-3′. The 3′-primer was designed to introduce an *Eco*RI site immediately 5′ of the translation initiation ATG of *GAP* (Fig. 1). The resulting vector, named pHWO10, is composed of the origin of replication and the ampicillin resistance gene (*amp*) from *E. coli* vector pBR322, the *P. pastoris HIS4* gene (Cregg et al., 1985), and the *P. pastoris AOX1* transcription termination sequence separated from P_{GAP} by a unique *Eco*RI site (Fig. 3). The vector is suitable for targeted integration into the genomic *HIS4* or P_{GAP} loci of *P. pastoris*.

2.4. Analysis of P_{GAP} and comparison to P_{AOX1}

To compare the relative strengths of P_{GAP} and P_{AOX1}, a 1.23-kb *Pst*I fragment from vector pPIC9K

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-493                                                                    ggatccttttttgtagaaa
-474  tgtcttggtgtcctcgtccaatcaggtagccatctctgaaatatctggctccgttgcaactccgaacgacctgctggca
-395  acgtaaaattctccgggggtaaaacttaaatgtggagtaatggaaccagaaacgtctcttccctctctctccttccacc
-316  gcccgttaccgtccctaggaaatttttactctgctggagagcttcttctacggccccttgcagcaatgctcttcccagc
-237  attacgttgcgggtaaaacggaagtcgtgtacccgacctagcagcccagggatggaaaagtcccggccgtcgctggcaa
-158  taatagcgggcggacgcatgtcatgagattattggaaaccaccagaatcgaatataaaaggcgaacacctttcccaatt
 -79  ttggtttctcctgacccaaagactttaaatttaatttatttgtccctatttcaatcaattgaacaactatcaaaacaca

   1  ATG GCT ATC ACT GTC GGT ATT AAC GGT TTC GGA CGT ATT GGA CGT CTC GTT CTG AGA GTC
   1   M   A   I   T   V   G   I   N   G   F   G   R   I   G   R   L   V   L   R   V

  61  GCT CTT TCC AGA GCT GAC ATC AAG GTT GTT GCT ATC AAC GAC CCA TTC ATT GCT CCA GAA
  21   A   L   S   R   A   D   I   K   V   V   A   I   N   D   P   F   I   A   P   E

 121  TAC GCT GCT TAC ATG TTC AAG TAC GAC TCT ACC CAC AAG GCT TAC AAG GGT GAG GTT TCT
  41   Y   A   A   Y   M   F   K   Y   D   S   T   H   K   A   Y   K   G   E   V   S

 181  GCC AGC GGC AAC AAG ATC AAC ATT GAC GGT AAA GAA ATC ACC GTT TTC CAA GAG AGA GAC
  61   A   S   G   N   K   I   N   I   D   G   K   E   I   T   V   F   Q   E   R   D

 242  CCT GTC AAC ATC CCA TGG GGT AAG GCT GGT GTC GAC TAC GTC ATT GAG TCC ACC GGT GTT
  81   P   V   N   I   P   W   G   K   A   G   V   D   Y   V   I   E   S   T   G   V

 301  TTC ACC ACT TTG GAG GGT GCC CAA AAG CAC ATC GAC GCC GGT GCC AAG AAG GTG GTC ATC
 101   F   T   T   L   E   G   A   Q   K   H   I   D   A   G   A   K   K   V   V   I

 363  ACT GCT CCA TCC AAG GAT GCT CCA ATG TTC GTT GTC GGT GTC AAC GAG GAG AAA TAC ACT
 121   T   A   P   S   K   D   A   P   M   F   V   V   G   V   N   E   E   K   Y   T

 421  TCT GAC TTG AAC ATT GTC TCC AAT GCT TCT TGT ACT ACC AAC TGT TTG GCT CCA TTG GCC
 141   S   D   L   N   I   V   S   N   A   S   C   T   T   N   C   L   A   P   L   A

 481  AAG GTT GTC AAC GAC ACT TTC GGA ATT GAG TCC GGT TTG ATG ACC ACC GTC CAC TCC ATG
 161   K   V   V   N   D   T   F   G   I   E   S   G   L   M   T   T   V   H   S   M

 541  ACC GCC ACT CAA AAG ACC GTT GAC GGT CCA TCC CAC AAG GAC TGG AGA GGT GGT AGA ACG
 181   T   A   T   Q   K   T   V   D   G   P   S   H   K   D   W   R   G   G   R   T

 601  GCT TCT GGT AAC ATC ATT CCA TCT TCC ACT GGT GCT GCT AAG GCC GTC GGT AAG GTT ATT
 201   A   S   G   N   I   I   P   S   S   T   G   A   A   K   A   V   G   K   V   I

 661  CCA GAA TTG AAC GGT AAG CTG ACC GGT TTG GCT TTC CGT GTC CCA ACC GTC GAT GTC TCC
 221   P   E   L   N   G   K   L   T   G   L   A   F   R   V   P   T   V   D   V   S

 721  GTT GTT GAC TTG ACC GTC AAC TTG AAG AAG GAG ACT ACC TAC GAG GAG ATC AAG TCT GTT
 241   V   V   D   L   T   V   N   L   K   K   E   T   T   Y   E   E   I   K   S   V

 781  ATC AAG GCT GCT TCC GAG GGT AAG CTC AAG GGT GTT TTG GGT TAC ACT GAA GAT GCC GTT
 261   I   K   A   A   S   E   G   K   L   K   G   V   L   G   Y   T   E   D   A   V

 841  GTC TCT TCT GAC TTC TTG GGT GAC GAG AGA TCC TCC ATC TTC GAC GCT TCT GCC GGT ATT
 281   V   S   S   D   F   L   G   D   E   R   S   S   I   F   D   A   S   A   G   I

 901  CAA TTG ACT CCA TCT TTC GTC AAG CTG ATC TCT TGG TAC GAC AAC GAG TAC GGT TAC TCC
 301   Q   L   T   P   S   F   V   K   L   I   S   W   Y   D   N   E   Y   G   Y   S

 961  ACC AGA GTC GTC GAC TTG TTG CAA CAC GTT GCT AAG GCT TAA tcgatttgtatgtgaaatagctg
 321   T   R   V   V   D   L   L   Q   H   V   A   K   A   *

1026  aaattcgaaaatttcattatggctgtatctactttagcgtattaggcatttgagcattggcttgaacaatgcgggctgt
1105  agtgtgtcaccaaagaaaccattcgggttcggatctggaagtcctcatcacgtgatgccgatctcgtgtattttatttt
1184  cagataacacctgaagacttttgggtcggaggactggctctttccgatcaaattggaatggaaaattgctcctctaata
1263  aaggggtgcccaacactctttgtaacacaggacacgtttattgctaactcgattgcattctttcctttcccacaaccgg
1342  atctggtctggtgacatctctcctgtccttatctaaa
```

Fig. 1. Nucleotide and deduced amino acid sequences of *P. pastoris GAP*. The sequence data are available from EMBL/GenBank/DDBJ under accession number U62648.

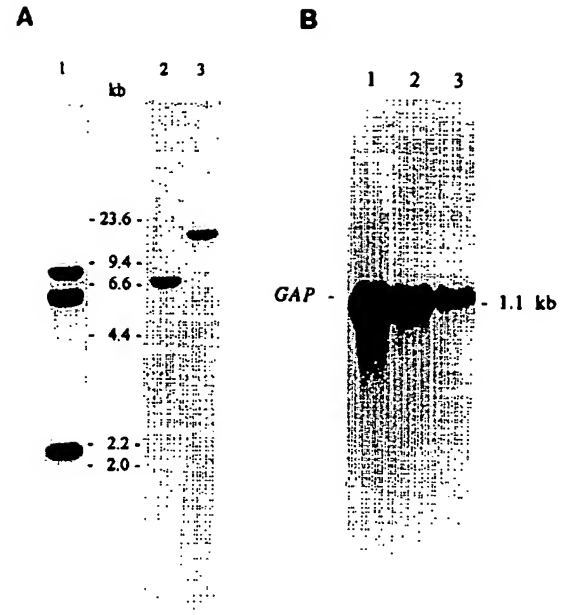

Fig. 2. (A) Southern blot analysis of genomic DNA isolated from *S. cerevisiae* (lane 1) or *P. pastoris* (lanes 2 and 3) using as a probe a 2.1-kb fragment containing the *S. cerevisiae TDH3* gene. **Methods:** Genomic DNA isolated from *S. cerevisiae* (lane 1, 0.5 μg) or *P. pastoris* (lanes 2 and 3, 1.0 μg) was digested with *Hin*dIII (lanes 1 and 2) or *Eco*RI (lane 3), transferred to nitrocellulose, and hybridized in the presence of 50% formamide to a *S. cerevisiae TDH3*-specific probe as described by Sambrook et al. (1989). (B) Northern blot analysis of total RNA from *P. pastoris* cells grown on different carbon sources using a *GAP*-specific probe. **Methods:** Total RNA was isolated from *P. pastoris* GS115 cells grown at 30°C in yeast nitrogen base medium supplemented with 50 mg/ml histidine using as carbon source 2% glucose (lane 1), 2% glycerol (lane 2), or 0.5% methanol (lane 3). Equal amounts of RNA (10 μg) were separated by formaldehyde-agarose electrophoresis, transferred to nitrocellulose and hybridized to a probe prepared from a 700-bp *Sal*I *GAP* fragment which encodes amino acids 91 through 324 from *P. pastoris* GAPDH (Fig. 1) as described by Sambrook et al. (1989).

containing the kanamycin resistance gene (Invitrogen, San Diego, CA) was first inserted into the *Pst*I site of the *amp* gene of vectors pHIL-A1 and pHWO10, resulting in vectors pHWO8 and pHWO9, respectively (Fig. 3). This insertion completely abolished β-lactamase activity derived from the *amp* gene in both *E. coli* and *P. pastoris*. As a reporter, a modified β-lactamase gene from *E. coli* (*bla*) was subcloned as a 0.83-kb *Eco*RI-*Hin*dIII^{klenow} fragment into *Eco*RI-*Age*I^{klenow}-digested pHWO8 and pHWO9 to make vectors pHWO18 (P_{AOX1}-*bla*), and pHWO19 (P_{GAP}-*bla*) (Fig. 3). The modification involved the removal of the amino-terminal secretion signal of β-lactamase to generate a reporter protein which remains cytoplasmic (Fig. 3). After linearization of both vectors with *Sal*I which is uniquely present in the *HIS4* gene, the vectors were integrated into the genomic *HIS4* locus of *P. pastoris* strain GS115 (*his4*) by electrotransformation (Becker and Guarente, 1991). Transformants were selected and analyzed by Southern hybridization to screen for ones containing a single copy of the vectors properly inserted at the *HIS4* loci (not shown).

Transformants containing pHWO18 or pHWO19 were grown in yeast nitrogen base medium supplied with either glucose, glycerol, methanol or oleic acid as sole carbon source. Oleic acid was included in this study because it is a commonly used carbon source to induce peroxisomes in *P. pastoris* (see below). Cells were harvested at the same density during mid-exponential growth phase ($OD_{600}=1.0$), and crude extracts were prepared and assayed for β-lactamase activity (Table 1). Samples were also examined on immunoblots using anti-β-lactamase antibodies to confirm that activity levels reflected the concentration of β-lactamase protein (not shown). As expected from the Northern blot analysis (Fig. 2B), β-lactamase was constitutively expressed under control of P_{GAP}, although its level varied significantly depending on the carbon source used for growth. The expression level was highest in glucose-grown cells and lowest in methanol-grown cells. β-Lactamase activity levels measured in extracts from cells grown on glucose, glycerol and methanol were generally proportional to those for GAPDH activity in the same extracts. However, in oleic acid-grown cells, β-lactamase activity was considerably lower than expected from the GAPDH activity measured in the same cells. The reason for this difference is unknown but could be due to the presence of additional regulatory sequences for response to oleic acid located 5′ or 3′ of the P_{GAP} fragment used in these studies.

The regulation of P_{AOX1} was previously described by Tschopp et al. (1987) using β-galactosidase as reporter. This study indicated that P_{AOX1} is subject to repression/induction regulation; i.e., in glucose- and glycerol-grown cells P_{AOX1} is fully repressed, whereas in methanol-grown cells the promoter is maximally induced. These findings were confirmed in our study using β-lactamase as a reporter, although extremely low levels of expression were detected on glucose and glycerol. P_{AOX1}-expression was low in extracts from oleic acid-grown cells and highest in extracts from methanol-grown cells (Table 1). A low level of expression from P_{AOX1} in oleic acid-grown cells has also been observed using firefly luciferase as a reporter (Spong and Subramani, 1993; Waterham et al., 1996).

Interestingly, P_{GAP}-controlled expression of *bla* in glucose-grown cells was significantly higher than P_{AOX1}-controlled expression of *bla* in methanol-grown cells. In addition, P_{GAP}-controlled expression of *bla* in glycerol-grown cells was approximately equal to P_{AOX1}-controlled expression of *bla* in methanol-grown cells (Table 1).

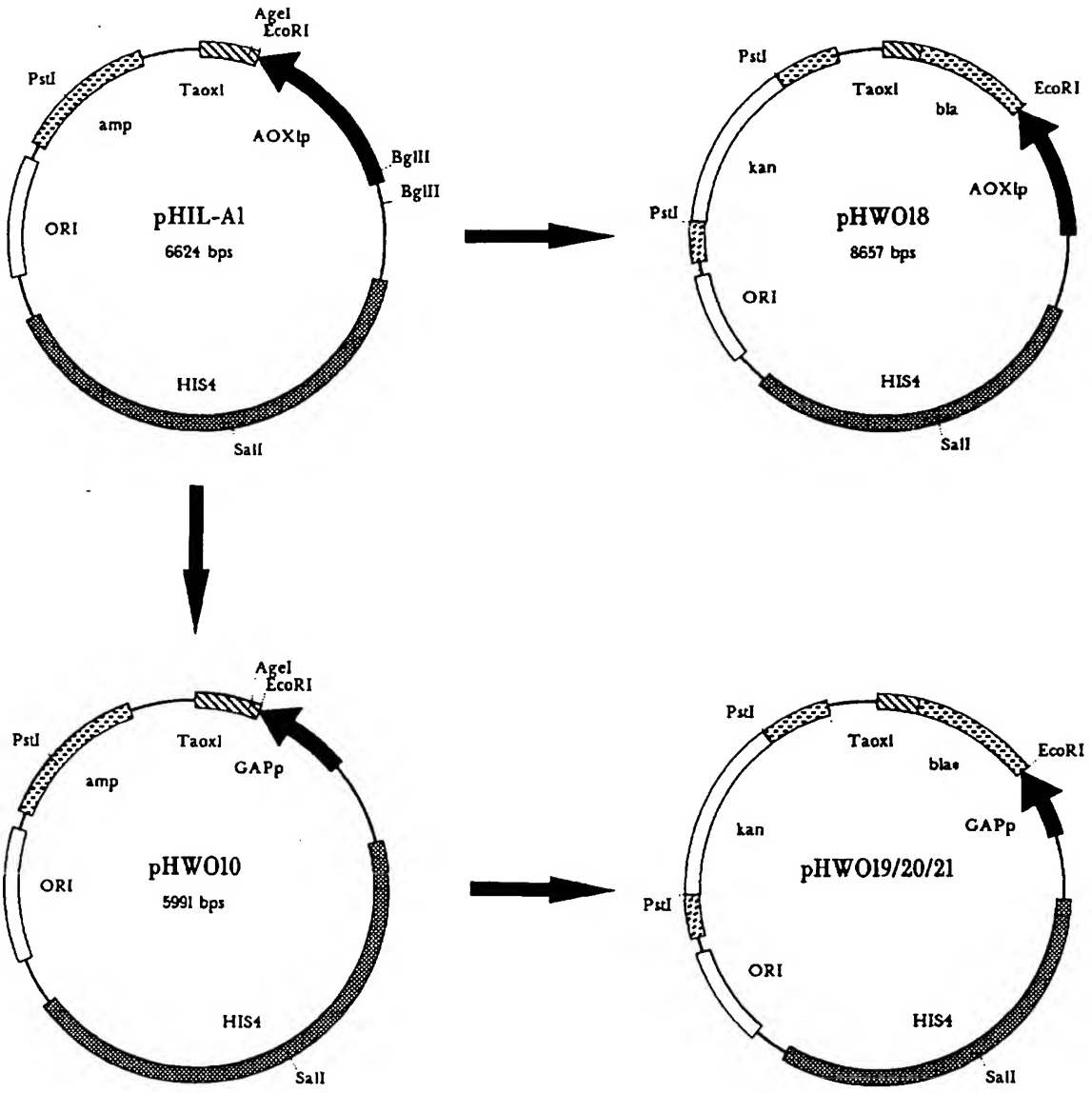

Fig. 3. Physical maps of selected vectors used in this study. **Methods:** The P_{GAP}-controlled expression vector pHWO10 was obtained from the P_{AOX1}-controlled expression vector pHIL-A1 (Invitrogen) by replacing the *Eco*RI-*Bgl*II P_{AOX1} fragment with an *Eco*RI-*Bam*HI P_{GAP} fragment generated by amplification by the polymerase chain reaction. For reporter studies, the *amp* genes from pHIL-A1 and pHWO10 were disrupted by insertion at their *Pst*I sites of a *Pst*I fragment from pPIC9K (Invitrogen) containing the kanamycin resistance gene to make vectors PHWO8 and PHWO9 (not shown). As a reporter for promoter studies, a modified *amp* gene (designated *bla*) encoding β-lactamase amino acids His^{24} through Trp^{286} (Sutcliffe, 1978) preceded by Met-Ser-Gly (Waterham et al., 1994) was inserted as an *Eco*RI-*Hin*dIII^{klenow} fragment at the *Eco*RI-*Age*I^{klenow} sites of pHWO8 and pHWO9 to make vectors pHWO18 and pHWO19. As a reporter for PTS1 protein import, the 3′-end of the *H. polymorpha PER1* gene encoding Per1p amino acids Asn^{642} through Leu^{650} was ligated in reading frame to the 3′-end of the *bla* gene encoding β-lactamase amino acids His^{24} through Trp^{286} (Sutcliffe, 1978) preceded by Met-Ser-Gly as described previously (Waterham et al., 1994). The resulting construct was inserted as an *Eco*RI-*Hin*dIII^{klenow} fragment at the *Eco*RI-*Age*I^{klenow} site of pHWO9 to make vector pHWO20. As a reporter for PTS2 protein import, the 5′-end of the watermelon malate dehydrogenase gene encoding amino acids Met^{1} through Met^{25} (Gietl, 1990) was amplified by the polymerase chain reaction and ligated in reading frame to the 5′-end of the *bla* gene encoding β-lactamase amino acids His^{24} through Trp^{286} (Sutcliffe, 1978). The resulting construct was inserted as an *Eco*RI-*Hin*dIII^{klenow} fragment at the *Eco*RI-*Age*I^{klenow} site of pHWO9 to make vector pHWO21.

2.5. *Use of* P_{GAP} *in a peroxisomal protein import study*

Peroxisomal proteins are encoded by nuclear genes and post-translationally imported into the organelles (Subramani, 1993; Waterham and Cregg, 1997). Two different peroxisomal targeting signals (PTS) have been identified that are responsible for correct delivery of most matrix proteins to the organelles. The first, PTS1, is a tripeptide with the consensus sequence Ser/Ala/Cys-Lys/Arg/His-Leu/Met found at the extreme carboxy

Table 1
β-Lactamase, GAPDH and AOX activities in total extracts of *P. pastoris* transformed with pHWO18 (P_{AOX1}-*bla*) and pHWO19[a] (P_{GAP}-*bla*) and grown on selected carbon sources[b]

Construct	Carbon	Enzyme activities[c]		
		β-Lactamase	GAPDH	AOX
P_{AOX1}-*bla*	Glucose	0.6 (0.08%)	5.8 (100%)	0.0 (0%)
	Glycerol	0.5 (0.06%)	4.5 (78%)	0.0 (0%)
	Oleic acid	1.3 (0.2%)	6.9 (119%)	0.7 (0.09%)
	Methanol	770.0 (100%)	2.0 (34%)	800.0 (100%)
P_{GAP}-*bla*	Glucose	1100.0 (100%)	6.6 (100%)	0.0 (0%)
	Glycerol	800.0 (73%)	4.6 (70%)	0.0 (0%)
	Oleic acid	500.0 (45%)	6.5 (98%)	1.2 (0.2)
	Methanol	400.0 (36%)	3.6 (55%)	800.0 (100%)

[a]Vectors pHWO18 (P_{AOX1}-controlled β-lactamase expression) and pHWO19 (P_{GAP}-controlled β-lactamase expression) were linearized with *SalI* and integrated into the genomic *HIS4* locus of *P. pastoris* strain GS115.

[b]Cells were grown in shake flasks at 30°C in yeast nitrogen base medium using as carbon source 0.5% glucose, 0.5% glycerol, 0.5% methanol or 0.2% oleic acid plus 0.05% Tween 40.

[c]Protein extracts were prepared from cells harvested at $OD_{600}=1.0$ using glass beads (Waterham et al., 1992). Protein concentrations were determined with the Pierce bicinchoninic acid protein assay kit (Rockford, IL, USA). AOX activity, expressed in nmol/mg per min (Verduyn et al., 1984) and GAPDH activity, expressed in mmol/mg per min (McAllister and Holland, 1985a) were assayed at 30°C. β-Lactamase activity, expressed as nmol/mg per min, was assayed spectrophotometrically at 569 nm and 30°C in 25 mM Tris-HCl (pH 7.5) using 10 mM 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium-methyl]-3-cephem-4-carboxylic acid (Calbiochem, La Jolla, CA, USA) as substrate (extinction coefficient 44.403 cm^{-1} M^{-1}). Activities represent the mean of two experiments.

terminus of many peroxisomal matrix proteins (de Hoop and AB, 1992). The second, PTS2, has the consensus sequence Arg/Lys-Leu/Ile-Xxx_5-His/Gln-Leu/Ala and is located near the amino terminus of a few peroxisomal proteins (Faber et al., 1995), including thiolase and watermelon glyoxysomal malate dehydrogenase (Gietl et al., 1994). To facilitate our studies on peroxisomal protein import in *P. pastoris*, we constructed two vectors using the modified β-lactamase as a reporter fused to either a PTS1 or a PTS2 sequence and expressed under control of P_{GAP}. β-Lactamase was chosen as a reporter because of the availability of a sensitive assay for its activity and specific antibodies against the protein, both of which facilitate its detection, and because it has been successfully used for this purpose in the related yeast *Hansenula polymorpha* (Waterham et al., 1994).

As a source for a PTS1 sequence, the 3′-end of the *H. polymorpha PER1* gene (Waterham et al., 1994) was ligated in reading frame to the 3′-end of the modified *bla* gene and inserted into vector pHWO9 to make vector pHWO20 (Fig. 3). Expression of this construct under control of P_{GAP} resulted in a β-lactamase-PTS1 hybrid protein composed of β-lactamase and the nine carboxy-terminal amino acids of Per1p, which terminates with the established PTS1 motif Ala-Lys-Leu (Waterham et al., 1994).

As a source for a PTS2 sequence, the 5′-end of the watermelon malate dehydrogenase gene (Gietl, 1990) was ligated in reading frame to the 5′-end of the modified *bla* gene and inserted into vector pHWO9 to make vector pHWO21 (Fig. 3). Expression of this construct under control of P_{GAP} resulted in a PTS2-β-lactamase hybrid protein composed of β-lactamase preceded by the first 25 amino-terminal amino acids of malate dehydrogenase, which includes the PTS2 motif Arg^{10}-Ile-Xxx_5-$His\text{-}Leu^{18}$ (Gietl, 1990; Gietl et al., 1994).

Vectors pHWO20 and pHWO21 were linearized with *SalI* and integrated into the *HIS4* locus of *P. pastoris* strain GS115. The locations of the β-lactamase-PTS1 and the PTS2-β-lactamase hybrid proteins were determined through subcellular fractionation of transformants grown on oleic acid to induce peroxisomes. After centrifugation of a postnuclear supernatant into a supernatant fraction, consisting primarily of cytosol, and an organelle pellet fraction, containing mostly peroxisomes and mitochondria, the major portion of both hybrid proteins was recovered in the organelle pellet. As a control, β-lactamase without either PTS was found in the cytosolic supernatant fraction (not shown). Subsequent sucrose density centrifugation of the organelle pellets indicated that both the β-lactamase-PTS1 (Fig. 4A) and the PTS2-β-lactamase (Fig. 4B) hybrid proteins were correctly targeted to peroxisomes since the distribution of β-lactamase activity across the gradients was similar to the distribution of the peroxisomal marker enzyme catalase and clearly different from the distribution of the mitochondrial marker enzyme cytochrome *c* oxidase.

2.6. General applications for P_{GAP}

P. pastoris is an important host organism for the production of heterologous proteins (Cregg et al., 1993). Expression of foreign genes in the yeast is typically under control of P_{AOX1}, whose tight regulation allows the introduction and maintenance of heterologous genes during repressing growth conditions to prevent selection for nonexpressing strains. However, in some instances it may not be suitable to use methanol for the induction of gene expression. For example, because methanol is most commonly derived from petrochemical sources, it may not be an acceptable carbon source for production of certain food products and additives. In addition, large volumes of flammable methanol are a potential hazard, whereas other carbon sources such as glucose or glycerol are not.

P_{GAP} is an attractive alternative to P_{AOX1} for the production of some heterologous proteins in *P. pastoris*; in glucose-grown shake-flask cultures, P_{GAP} appears to

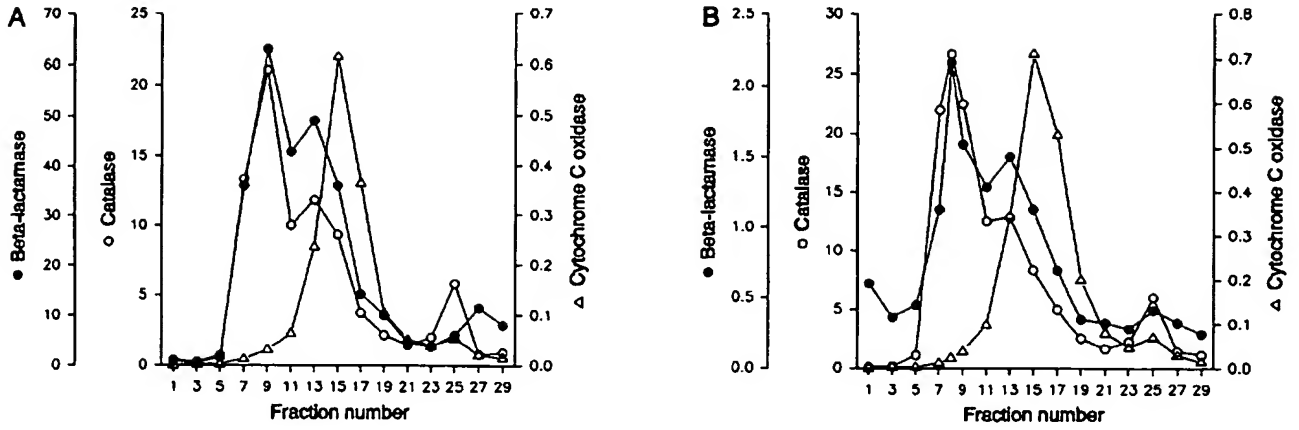

Fig. 4. Sucrose density gradient profiles obtained after subcellular fractionation of oleic acid-grown *P. pastoris* transformants expressing β-lactamase-PTS1 (A) or PTS2-β-lactamase (B) under transcriptional control of P_{GAP}. **Methods:** Vectors pHWO20 expressing β-lactamase-PTS1 and pHWO21 expressing PTS2-β-lactamase under transcriptional control of P_{GAP} were linearized with *SalI* and integrated at the *HIS4* locus of *P. pastoris* GS115. Transformed cells were grown at 30°C in yeast nitrogen base medium using as carbon source 0.2% oleic acid plus 0.05% Tween 40, and subjected to subcellular fractionation by differential centrifugation as described previously (Waterham et al., 1996). The 30 000 × *g* organelle pellet which consists mainly of peroxisomes and mitochondria was further fractionated on a sucrose density gradient, and 1-ml fractions were collected from the bottom of the gradient (Waterham et al., 1996). Fractions were assayed for peroxisomal catalase (U/ml; Lück, 1963), mitochondrial cytochrome *c* oxidase (U/ml; Douma et al., 1985) and β-lactamase activities (U/ml; see legend of Table 1).

be significantly stronger than P_{AOX1} in methanol-grown shake-flask cultures. Using the P_{GAP}-based vector pHWO10, we have successfully expressed genes from bacteria (*amp*, *bla*, *lacZ*), yeast (*AOX1* and *PER6*), plants (*gMDH*), insects (luciferase gene) and mammals (*PAF1*, *CPT1* and *CPTII*) in *P. pastoris* following the strategy outlined for the β-lactamase constructs.

3. Conclusions

(1) *P. pastoris* contains a single *GAP* gene that is predicted to encode a 35.4-kDa protein (333 amino acids) with strong similarity to other eukaryotic and prokaryotic GAPDHs.

(2) A *P. pastoris* integrative expression vector, pHWO10, has been constructed which expresses foreign genes under control of a 500-bp P_{GAP} fragment. The P_{GAP} sequence is separated from the transcription termination sequence of the *P. pastoris AOX1* gene by a unique *Eco*RI site suitable for the insertion of foreign genes. In addition, the vector contains the *P. pastoris HIS4* gene, and the ampicillin resistance gene and origin of replication from *E. coli* vector pBR322.

(3) P_{GAP} is a strong and constitutive promoter, although its strength varies depending on the carbon source used for cell growth. Expression under control of P_{GAP} in glucose-grown *P. pastoris* cells is higher than that of the commonly used P_{AOX1} in methanol-grown cells, which makes P_{GAP} an attractive alternative for production of heterologous proteins in *P. pastoris*.

(4) β-Lactamase is a suitable reporter protein for peroxisomal protein import studies in *P. pastoris*. β-Lactamase-PTS1 and PTS2-β-lactamase hybrid proteins expressed under control of P_{GAP} are correctly targeted to wild-type *P. pastoris* peroxisomes.

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Modeling *Pichia pastoris* Growth on Methanol and Optimizing the Production of a Recombinant Protein, the Heavy-Chain Fragment C of Botulinum Neurotoxin, Serotype A

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Abstract: An unstructured growth model for the recombinant methylotrophic yeast *P. pastoris* Mut$^+$ expressing the heavy-chain fragment C of botulinum neurotoxin serotype A [BoNT/A(H_c)], was successfully established in quasi-steady state fed-batch fermentations with varying cell densities. The model describes the relationships between specific growth rate and methanol concentration, and the relationships between specific methanol and ammonium consumption rates and specific growth rate under methanol-limited growth conditions. The maximum specific growth rate (μ) determined from the model was 0.08 h^{-1} at a methanol concentration of 3.65 g/L, while the actual maximum μ was 0.0709 h^{-1}. The maximum specific methanol consumption rate was 0.0682 g/g WCW/h. From the model, growth can be defined as either methanol-limited or methanol-inhibited and is delineated at a methanol concentration of 3.65 g/L. Under inhibited conditions, the observed biomass yield ($Y_{X/MeOH}$) was lower and the maintenance coefficient (m_{MeOH}) was higher than compared to limited methanol conditions. The $Y_{X/MeOH}$ decreased and m_{MeOH} increased with increasing methanol concentration under methanol-inhibited conditions. BoNT/A(H_c) content in cells (α) under inhibited growth was lower than that under limited growth, and decreased with increasing methanol concentration. A maximum α of 1.72 mg/g WCW was achieved at a μ of 0.0267 h^{-1} and induction time of 12 h.
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Keywords: *Pichia pastoris;* fed-batch; growth modeling; optimization; fermentation; botulinum neurotoxin

INTRODUCTION

The *Pichia pastoris* expression system has been used extensively for the production of heterologous proteins. The advantages of this system include high cell densities on a defined minimal basal salts medium (Brierley et al., 1990; Cregg and Higgins, 1995), efficient post-translational modifications (Digan et al., 1988; Tschopp et al., 1987), less secretion of endogenous proteins while expressing secreted recombinant protein (Digan et al., 1988; Laroche et al., 1994; Tschopp et al., 1987), and a strong, well-regulated methanol-induced promoter (Cregg and Madden, 1988; Cregg and Vedvick, 1993). It is known that *P. pastoris* can assimilate methanol but cannot tolerate high methanol concentrations. This is due to the accumulation of formaldehyde and hydrogen peroxide inside the cells, both of which are the oxidized products of methanol by the alcohol oxidase and are toxic to the cell (Couderc and Baratti, 1980; Cregg and Madden, 1988; Van der Klei et al., 1990). In order to obtain high cell densities while maintaining a low level of methanol, a fed-batch strategy is commonly used. The methanol feeding strategy, which also dictates the specific growth rate, is one of the most important factors for maximizing recombinant protein production, since all of the biochemical reactions for product formation are directly or indirectly associated with cell growth (Shioya, 1992).

The methanol feeding strategy used by most researchers for methanol utilization positive (Mut$^+$) recombinant *P. pastoris* fed-batch fermentation is from "Pichia Fermentation Process Guidelines" of Invitrogen Co. (San Diego, CA). There are two different empirical feeding strategies that are commonly used (Stratton et al., 1998). The first is the dissolved oxygen (D.O.) spike method, where during methanol induction the methanol feed pump is stopped and the amount of time is recorded for the D.O. to increase 10% (a limited culture will have D.O. spike times of 15–30 s). The second method uses preprogrammed linear feed rates that typically do not exceed 11 mL/L/h. Both protocols are designed to maintain a residual methanol concentration of nearly 0 g/L, which is not necessarily optimum for maxi-

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mum protein production. Some protocols for regulating methanol feed rate were reported in the production of bovine lysozyme (Brierley et al., 1990), but the effect of methanol feed rate on the protein production was not detailed. Shake flask cultures with methanol maintained at 0.3% v/v were studied by using a methanol sensor (Guarna et al., 1997). The results showed the volumetric protein productivity, while maintaining a constant methanol level, increased 5-fold over cultures that were not maintained with constant methanol. Because the specific growth rate and specific production rate were not compared, it is hard to conclude if the methanol affected the protein production rate. The specific growth rate and specific protein production rate were determined with methanol concentrations ranging between 1.5 and 31 g/L (Katakura et al., 1998), but the study did not extend to methanol-limited growth or develop a growth model.

The purpose of this research is to establish an unstructured growth model for *P. pastoris* Mut$^+$ and use the model to optimize the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A [BoNT/A(H_c)]. The botulinum neurotoxin is produced by *Clostridium botulinum* under anaerobic conditions and is classified into seven serotypes designated types A through G. It is the most potent toxin known to science (Lamanna, 1959). BoNTA/(H_c), the 50-kDa carboxyl-terminal region of the botulinum neurotoxin, is involved in the recognition of specific ectoreceptors on peripheral cholinergic nerve cells. The toxin fragment is nontoxic and has been shown to elicit significant protective immunity in mice and is a vaccine candidate (Byrne et al., 1998; Clayton et al., 1995).

MATERIALS AND METHODS

Experimental Setup

Fermentations were performed with a 5-L Bioflo 3000 fermentor interfaced with the computer-based software, AFS-BioCommand, for data acquisition and supervisory control (New Brunswick Scientific Co., Edison, GA). A part of the off-gas was diverted to an MC-168 methanol monitor and controller (PTI Instruments, Inc., Kathleen, GA) equipped with a TGS822 methanol sensor (Figaro Engineering Inc., Osaka, Japan), which were used to maintain a constant level of methanol in the broth. A methanol feed pump (Model 101U/R, Watson-Marlow Ltd., England), balance (Model PR1203, Mettler Toledo, Switzerland), and the MC-168 controller were interfaced with the AFS-BioCommand to make a closed-loop control system, Fig. 1. The D.O. was set to 20% and controlled by an agitation/O_2 cascade available as part of the BioFlo 3000 system. Pure oxygen was supplied as needed to maintain 20% of D.O. saturation.

Strain and Inoculum Preparation

P. pastoris GS115 Mut$^+$, expressing the BoNT/A(H_c) fragment intracellularly, is described elsewhere (Byrne et al., 1998). Attempts were made to secrete the BoNT/A(H_c) fragment, but the fragment was glycosylated, which rendered the fragment immunologically inactive. The native toxin is not glycosylated. The gene was determined to be stable after a minimum of 25 generations (data not shown). Individual fermentors were inoculated with 40 mL of freshly thawed starter culture. These cultures were prepared by inoculating 200 mL of BMGY (buffered minimal glycerol complex medium, *Pichia* Expression Kit, Invitrogen Co.) containing 10 g/L glycerol in a 2-L baffled shake flask with 1 mL of a stock culture maintained frozen in liquid nitrogen (vapor phase). The culture was incubated at 30°C with 200-rpm shaking rate until an OD_{600} (optical density at 600 nm) between 2 and 6 was reached. The entire 200 mL was aseptically transferred to a 5-L fermentor containing 4 L of BMGY with 40 g/L glycerol. When the density reached an OD_{600} of 50, the cells were harvested aseptically using sterile 500-mL centrifuge bottles and centrifuged at 2000*g*. The pellets were resuspended in 1 L of sterilized BMGY containing 150 g/L glycerol. Cell suspension aliquots of 40 mL (approximately 200 OD_{600}) were dispensed into 50-mL sterile conical tubes and stored at −80°C until ready for use.

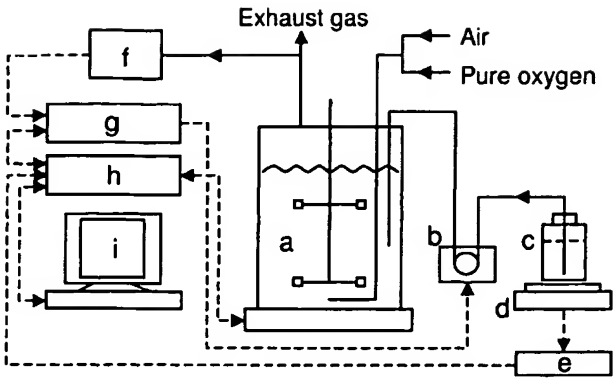

Figure 1. Schematic diagram of fermentation control system. (→) Mass flow direction; (⋯→) controlling signal flow direction; (a) fermentor; (b) methanol feed pump; (c) methanol reservoir; (d) balance; (e) NBS scale interface; (f) MC-168 methanol controller; (g) NBS I/O converter; (h) BioCommand interface; (i) PC, AFS-BioCommand software.

Fermentation

Glycerol Batch Phase

The batch phase was 2 L of basal salts medium (BSM), which, per liter, consists of 26.7 mL 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, and 40.0 g glycerol. Prior to inoculation, the pH was adjusted to 5.0 with concentrated ammonium hydroxide followed by the addition of 8.7 mL of PTM1 trace salts (containing, per liter, 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin, and 5.0 mL H_2SO_4). Batch phase conditions were tempera-

ture, 30°C; pH, 5.0 (controlled with concentrated ammonium hydroxide); and D.O., 20%. These variables were monitored and maintained continuously by the fermentation unit. The end of batch phase was indicated by a spike in the D.O. caused by the exhaustion of glycerol. The cell mass at the end of the batch phase was approximately 100 g/L wet cells.

Glycerol Fed-Batch Phase and Transition Phase

The fed-batch phase was performed under glycerol-limited conditions to increase cell mass and prepare the cells for induction. Limited feed benefits the induction of the AOX1 (alcohol oxidase) promoter by facilitating the consumption of metabolites, such as acetate and ethanol, which build up during the batch phase and are inhibitory to AOX1 induction. The suggested minimum length of time for the fed-batch phase is 1 h. Longer times and/or higher feed rates may be used to increase cell mass prior to induction. For this project a 1-h fed-batch period was used at a feed rate of 20 g 50% w/w glycerol (containing 12 mL of PTM1 per liter) per hour per liter of broth.

Following the fed-batch phase, a transition phase was designed to shorten the time required for the cells to fully adapt to methanol. The transition phase is initiated by the addition of 1.5 g/L methanol, which initiates induction. The glycerol feed rate is simultaneously set to ramp down linearly from 20 g/L/h to 0 over a 3-h period. During the first hour, the cells continued to utilize glycerol as the primary carbon and energy source, which was confirmed by off-gas analysis. After the first hour the methanol concentration was observed to decrease quickly until by the end of the second hour the detection limit of the methanol sensor was reached. These two hours were defined as the transition phase, during which the cells initiated the switch from glycerol to methanol. By the end of the transition phase the cells were fully adapted to methanol, which was confirmed by a sharp drop in the D.O. when the methanol feeding profile was initiated. This two-hour adaptation time compares to a period of 4–5 h when using the traditional method of complete glycerol exhaustion followed by a very low methanol feed (Stratton et al., 1998).

Methanol Fed-Batch Phase

Once the cells were fully transitioned, the methanol (containing 12 mL of PTM1 per liter) was fed to start the methanol fed-batch phase (production phase). Two feeding strategies were investigated. The first was a methanol-excess feed strategy in which the methanol concentration was controlled at set levels between 2.0 and 30.0 g/L to study the effects of excess methanol on growth and protein production. The rate of increasing the methanol concentration was limited to 10 g/L/h to prevent the cells from being shocked. This strategy allowed the maximum specific growth rate, μ_m, to be determined. The second strategy controlled the growth rate below μ_m by methanol-limited feeding. The specific growth rate was kept at desired values by controlling the methanol feed rate at an exponential increase, which allowed the growth, production, and substrate consumption rates to be determined.

Analytical Methods

Cell density was expressed as wet cell weight (WCW), which was measured by removing duplicate 10-mL aliquots of the fermentation broth into preweighed 15-mL conical tubes. The samples were centrifuged at 2000*g*, the supernatants were decanted, and the pellets were weighed. One gram of wet cells is equivalent to approximately 0.28 g dry cell weight.

The off-line methanol concentration was determined by gas chromatography (GC-17A, Shimadzu Co., Columbia, MD) with isopropyl alcohol as an internal standard.

Cell were disrupted as a 150 g WCW/L suspension of cells in lysis buffer [2.92 g NaCl, 1.86 g EDTA, 10.47 g MOPS, sodium (Calbiochem Co., San Diego, CA), 2.5 g CHAPS (Pierce Co., Rockford, IL), in 1 L ddH_2O, pH to 7.5 with NaOH]. Aliquots of 1 mL were mixed with approximately 2.2 g zirconia/silica beads (Biospec Products, Inc. Bartlesville, OK) in 2.0-mL screw-cap tubes followed by disruption at 4°C with a vibrating disrupter (Mini-BeadBeater-8, Biospec Products, Inc.) for 8 cycles (1 min vibrating and 4 min resting in each cycle). The lysate/bead mixture was centrifuged until the supernatant was clear, and 100 μL aliquots were diluted 20-fold with lysis buffer containing 0.5 g/L CHAPS and then stored frozen at −20°C until analyzed for BoNT/A(H_c).

BoNT/A(H_c) was determined by using a modified ELISA procedure (Byrne et al., 1998). All incubations were completed at 37°C with sealed plate covers unless indicated differently. Microtiter plates (Immulon 4HBX; Dynex Technologies Inc., Chantilly, VA) were incubated overnight at 4°C with 100 μL per well of coating monoclonal antibody 5BA2 (Hallis et al., 1993) at 0.5 μg/mL in 100 mM sodium carbonate buffer, pH 9.6 (SCB). The plates were then blocked for 2 h with 5% w/v nonfat dry milk in SCB. The plates were washed with SCB followed by the addition of 100 μL of either sample or standard to each well and were then incubated for 90 min. Lysate samples were diluted 1/1000 to 1/2000 in SCB which contained 5% w/v nonfat dry milk and 0.05% w/v CHAPS before addition to the plates. The plates were washed with SCB then incubated for 1 h after the addition of 100 μL per well of buffered affinity-purified horse anti-BoNT/A(H_c) at 2.0 μg/mL in 5% w/v nonfat dry milk in phosphate-buffered saline with 0.05% w/v Tween 20 (PBS-T). The wash solution was changed to PBS-T. The plates were washed and incubated for 1 h with 100 μL per well of peroxidase-labeled goat anti-horse IgG [H+L] (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 2.0 μg/mL in 5% w/v nonfat dry milk in PBS-T. The plates were washed again and 100 μL of ABTS [2,2′-azinobis(3-ethlybenzthiazoline-6-sulfonic acid)] peroxidase substrate (Kirkegaard & Perry Laborato-

ries, Gaithersburg, MD) was added to each well followed immediately by incubation at room temperature (for 10–30 min) until the A_{405} of the highest standard was near 2. The reaction was stopped by addition of 100 μL of 2% w/v oxalic acid in water. The A_{405} was read with a MRX microplate reader (Dynex Technologies Inc.).

Calculation of the Specific Rates

The specific growth rate, μ, for a fed-batch culture is expressed as:

$$\mu = \frac{d(XV)}{(XV)dt}, \tag{1}$$

where X, V, and t are cell density, culture volume, and time, respectively, and μ is determined from the slope of $\ln(XV)$ versus t. At each sampling point t, V is determined from the sum of the initial broth volume, the volume of ammonium hydroxide, glycerol, and methanol added up to that time, minus the volume sampled.

If the methanol concentration and pH are constant from t_0 to t, the specific methanol (ν_{MeOH}) and ammonium (ν_{NH_3}) consumption rates, and the specific BoNT/A(H_c) production rate, ρ, are expressed as (Katakura et al., 1998; Omasa et al., 1992a,b)

$$Q_{MeOH} = \nu_{MeOH} \int_{t_0}^{t} (XV)dt, \tag{2}$$

$$Q_{NH_3} = \nu_{NH_3} \int_{t_0}^{t} (XV)dt, \tag{3}$$

$$J = \rho \int_{t_0}^{t} (XV)dt, \tag{4}$$

where Q_{MeOH} and Q_{NH_3} are the total amount of methanol and ammonium consumed, respectively, and J is the total protein produced from t_0 to t. Q_{MeOH} and Q_{NH_3} are determined by measuring the amount of methanol and ammonium added to the system. If μ is constant from t_0 to t, and, $X = X_0$ and $V = V_0$ at $t = t_0$, the following equations are obtained:

$$XV = X_0V_0e^{\mu(t-t_0)}, \tag{5}$$

$$\int_{t_0}^{t} (XV)dt = (XV - X_0V_0)/\mu. \tag{6}$$

When Eq. (6) is combined with Eqs. (2) and (3), ν_{MeOH} and ν_{NH_3} are obtained from the slope of Q_{MeOH} versus $(XV - X_0V_0)/\mu$ and Q_{NH_3} versus $(XV - X_0V_0)/\mu$, respectively. J is determined from

$$J = \alpha(XV) - \alpha_0(X_0V_0), \tag{7}$$

where α is the BoNT/A(H_c) content in the cells, as determined by ELISA, and $\alpha = \alpha_0$ at $t = t_0$. If α is constant during the fed-batch culture at a constant μ from t_0 to t, Eq. (7) becomes

$$J = \alpha(XV - X_0V_0). \tag{8}$$

When Eqs. (4), (6), and (8) are combined, ρ is

$$\rho = \alpha\mu. \tag{9}$$

MODELING OF THE FED-BATCH GROWTH

A CSTR or exponential fed-batch culture can operate as a chemostat when the substrate feed concentration, S_f, satisfies the following equation (Blanch and Clark, 1996):

$$S_f = S + \frac{X}{Y_{X/S}}, \tag{10}$$

where $Y_{X/S}$ is the observed yield of biomass to substrate. Both $Y_{X/S}$ and S are dependent upon the desired μ and the type of substrate, so X is decided by S_f for a desired μ. Since the maximum X is limited to a certain level based on a given medium and fermentor design, i.e., oxygen supply, heat transfer, etc., the maximum S_f is also limited.

It is proposed to model the fed-batch culture as a quasi-steady state system, where μ and S are constant, and X varies within a limited range. This is possible if the biochemical activities of the cell do not vary significantly over the range of the cell densities being studied, then μ, ν, and ρ will be constant. Therefore, a quasi-steady state will be defined as a growth condition where X varies while μ, ν, ρ, and S are constant within an experimental range of cell densities.

Maintaining a constant μ is essential for attaining a quasi-steady state. μ is a function of S, and S can be maintained constant over a range of 1–30 g/L using a methanol sensor. When the methanol concentration is below the sensor's limit of detection, an alternative means of maintaining a constant μ is required. In this case, the methanol feed rate (F) varies exponentially to maintain a constant μ using the following equation:

$$F = \nu_{MeOH}(X_0V_0)e^{\mu t}, \tag{11}$$

where X_0 is the cell density and V_0 is the broth volume when initiating the feed profile ($t = 0$). Once μ_m and $\nu_{MeOH,m}$ have been determined empirically under methanol excess conditions, ν_{MeOH} is estimated for a desired μ by the following equation:

$$\nu_{MeOH} = \mu\nu_{MeOH,m}/\mu_m. \tag{12}$$

Eq. (12) is based on an assumption that $Y_{X/S} = \mu_m/\nu_{MeOH,m}$ and is independent of μ and that the maintenance coefficient is negligible. This assumption is false, but it allows for an initial estimation of ν_{MeOH}. Eq. (12) will be revised later to give the true relationship between μ and ν_{MeOH}. When Eq. (12) is substituted into Eq. (11), F is estimated to deliver a constant desired μ. Although the actual values of μ and ν_{MeOH} will differ from the estimated values, it is only important that they are constant. If μ and ν_{MeOH} are constant, a quasi-steady state is attained.

Once the values of S, ν_{MeOH}, ν_{NH_3}, and α in the quasi-steady state with different desired μ have been determined by using either limited or excess feeding, an unstructured

growth model which describes the relationships between μ and S, ν_{MeOH}, and μ, and ν_{NH_3} and μ can be established. The production model, which describes the relationship between μ and α or between μ and ρ, is also achieved. From these two models all of the parameters related to growth and production can be predicted.

RESULTS AND DISCUSSION

Prior to this work, optimization of the methanol feeding strategy was viewed as empirical, following the standard procedure using either the D.O. spike method of methanol feeding (Brierley et al., 1990) or preprogrammed linear feed rates (Stratton et al., 1998). Even though the D.O. spike method was used to successfully produce protein, the method is inconvenient and subjects a high-cell density culture to changes in methanol feeding during D.O. spikes. The standard procedure is to reach a certain methanol-feeding rate, i.e., 11 mL/L/h and maintain this rate for the duration of the fermentation, which could sometimes be 36–70 h. Thus, as the biomass increased, the amount of methanol being fed per unit cell mass was decreasing. Since the production of recombinant protein and growth are regulated by the same type of promotor, our approach was to develop a model that coupled growth rate to product formation.

Establishing the Growth Model

Methanol-Nonlimited Cultures

To determine the growth characteristics of the culture when methanol is in excess, fermentations were performed with S between 2.0 and 30.0 g/L. The methanol concentrations were maintained at the desired levels throughout the fermentations with only minor fluctuations. Exponential growth was maintained at all levels of methanol, and this demonstrates that a quasi-steady state was maintained. As the S increased, μ decreased, which shows the inhibitory effects of methanol at high concentrations, Fig. 2. From the completed fermentations, μ_m and $\nu_{MeOH,m}$ were found to be 0.0709 h^{-1} and 0.0682 g/g WCW/h, respectively, when S was 2.1 g/L.

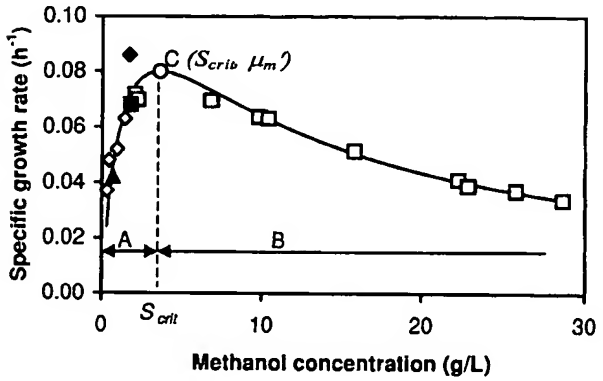

Figure 2. Relationship between specific growth rate and methanol concentration. (□) Methanol non-limited cultures; (◇) methanol-limited cultures; (◆, ■, ▲) cultures of *P. pastoris* wild type Y-11430, host strain GS115 his$^-$ and GS115 his$^+$ BoNT/E(H_c) clone, respectively.

Methanol-Limited Cultures

When μ_m and $\nu_{MeOH,m}$ are substituted into Eqs. (11) and (12), the methanol feed rate, F, was estimated by the following equation:

$$F = 0.962\mu(X_0V_0)e^{\mu t}. \quad (13)$$

The desired values for μ were set to 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 h^{-1}, and X_0 and V_0 were determined just prior to the start of the feed profile. The actual μ values were 0.0112, 0.0186, 0.0267, 0.0372, 0.0481, and 0.0630 h^{-1}, which correlated well to the desired values. The difference between the predicted μ and the actual μ is attributed to the error in estimating ν_{MeOH} by Eq. (12). When the resulting μ was 0.0112, 0.0186, and 0.0267 h^{-1}, S was below the GC detection limit. When μ was 0.0372, 0.0481, 0.052, and 0.064 h^{-1}, the S was 0.336, 0.454, 0.971, and 1.265 g/L, respectively. These values of μ and S were also plotted in Fig. 2 together with the values obtained in the nonlimited cultures.

Growth Model

The data of μ versus S fit the following uncompetitive inhibition growth model (Blanch and Clark, 1996):

$$\mu = \frac{0.146S}{1.5 + S + S^2/8.86}. \quad (14)$$

The solid line in Fig. 2 is the predicted value of μ based on Eq. (14), which shows that μ increased with S up to a maximum value, S_{crit}, beyond which it declines. The theoretical maximum specific growth rate, μ_m', at point C (Fig. 2), and S_{crit} are 0.08 h^{-1} and 3.65 g/L, respectively. The growth characteristics were divided into two regions based upon μ_m'. To the left of this point (region A) is the growth-limited region ($S < S_{crit}$), and to the right of point C (region B) is the growth-inhibited region ($S > S_{crit}$).

Simulation of Methanol-Limited Fed-Batch Cultures

Eq. (14) does not completely simulate a fed-batch culture because it does not account for the relationships between ν_{MeOH} and μ and ν_{NH_3} and μ, which can be expressed as (Blanch and Clark, 1996)

$$\nu = \frac{\mu}{Y_{X/S,t}} + m. \quad (15)$$

The relationship between ν_{MeOH} and μ, and ν_{NH_3} and μ under quasi-steady state limited conditions, Fig. 3, resulted in the following linear equations:

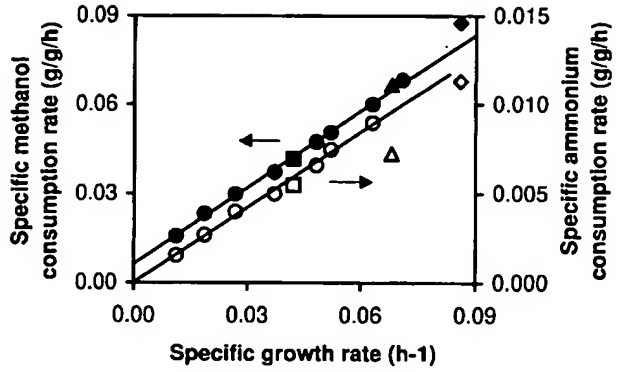

Figure 3. Relationship between specific substrate consumption rate and specific growth rate. (◆, ◇), (△, ▲), and (□, ■) are from cultures of *P. pastoris* wild type Y-11430, host strain GS115 his$^-$ and GS115 his$^+$ BoNT/E(H_c) clone, respectively.

$$\nu_{MeOH} = 0.84\mu + 0.0071, \quad (16)$$

$$\nu_{NH_3} = 0.14\mu. \quad (17)$$

The true yield of biomass and the maintenance coefficient, $Y_{X/S,t}$ and m with respect to methanol and ammonium, were $Y_{X/MeOH,t}$ = 1.19 g WCW/g MeOH, m_{MeOH} = 0.0071 g MeOH/g WCW/h; Y_{X/NH_3},t = 7.14 g WCW/g 28% NH_3, m_{NH_3} = 0, respectively.

Substitution of Eq. (16) into Eq. (11) results in the following equation:

$$F = (0.84\mu + 0.0071)(X_0V_0)e^{\mu t}, \quad (18)$$

which provides the corrected value of F for a methanol-limited fed-batch culture at a constant μ.

The following equations (19–24) were derived on the basis of the feeding strategy described by Eq. (18). In this simulation, $\mu < \mu_m' = 0.08\ h^{-1}$, $S < 3.65$ g/L, and $X < 450$ g WCW/L which was found to be the maximum X that the medium of BSM supplemented with PTM1 trace salts can support in a quasi-steady state.

$$XV = (X_0V_0)e^{\mu t}, \quad (19)$$

$$V_{MeOH} = (0.84\mu + 0.0071)(X_0V_0)(e^{\mu t} - 1)/0.79\mu, \quad (20)$$

$$V_{NH_3} = 0.14(X_0V_0)(e^{\mu t} - 1)/0.9, \quad (21)$$

$$V = V_0 + V_{MeOH} + V_{NH_3}, \quad (22)$$

$$X = \mu X_0 e^{\mu t}/[\mu + (1.22\mu + 0.009)X_0(e^{\mu t} - 1)], \quad (23)$$

$$S = f(\mu)\left(\text{rewritten } \mu = \frac{0.146S}{S + 1.5 + S^2/8.86}\right). \quad (24)$$

To confirm if the model is applicable to other strains, the fed-batch cultivation of *P. pastoris* wild type NRRL Y-11430, host strain GS115 his$^-$ (supplemented with 1 g/L histidine) and a GS115 his$^+$ BoNT/E(H_c) clone were synthesized. The actual values of S, μ, ν_{MeOH}, and ν_{NH_3} were within experimental agreement with the prediction. Therefore, the model could be applied to all the *P. pastoris* Mut$^+$ strains as a starting point, provided that protein expression does not significantly change the growth properties of the strain. It had been reported that the recombinant protein expression could change the strain bioactivities and make the substrate metabolism much different from the host strain (Katakura et al., 1998; Wong et al., 1998). In this case, the model should be modified on the basis of the individual protein properties.

$Y_{X/MeOH,obs}$ and m_{MeOH} During Inhibited Growth

For fed-batch fermentations under inhibited growth ($S > S_{crit}$), the effect of S on m_{MeOH} and the observed yield, $Y_{X/MeOH,obs} = \mu/\nu_{MeOH}$, were determined. The relationship between ν and μ during inhibited growth under quasi-steady state conditions was determined to be nonlinear, Fig. 4. It was observed that when S increased, $Y_{X/MeOH,obs}$ decreased and m_{MeOH} increased. From Eq. (16), the following equation was derived for calculating $Y_{X/MeOH,obs}$:

$$Y_{X/MeOH,obs} = \mu/\nu = 1.19 - 0.010/(0.085 + \mu). \quad (25)$$

The broken line A in Fig. 4 shows the predicted values of $Y_{X/MeOH,obs}$ calculated by substitution of Eq. (14) into Eq. (25), and the broken line B is the m_{MeOH} obtained in limited cultures. It was observed that the $Y_{X/MeOH,obs}$ was lower, while m_{MeOH} was higher compared to limited cultures when $S > 10$ g/L, illustrating a lower efficiency for cell mass synthesis at higher S.

Protein Production

A typical protein production time course during a fed-batch culture is presented in Fig. 5. α reached a maximum after 12 h post-induction and remained constant or decreased slightly until the end of the quasi-steady state, which demonstrates that quasi-steady state for α or ρ was not reached until BoNT/A(H_c) inside the cells reached a maximum. After X reached 400–450 g WCW/L, α started to decrease quickly, which confirmed the assumption that quasi-steady state exists within a certain range of X. The production

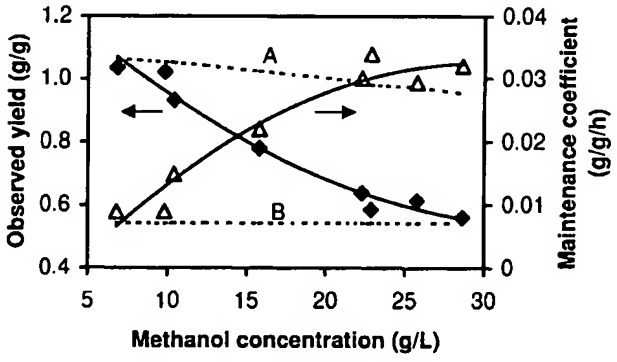

Figure 4. Effect of methanol concentration on observed yield of biomass and maintenance coefficient based on methanol in methanol-inhibited fermentations. (A) Observed yield calculated by substitution of Eq. (14) into Eq. (25). (B) Maintenance coefficient, m (0.0071), in the methanol-limited cultures.

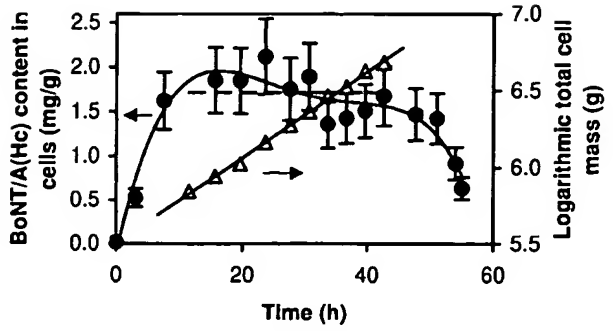

Figure 5. Typical time course of BoNT/A(H_c) production. Broken line: average value of BoNT/A(H_c) content in cells during the quasi-steady state.

level, α, shown in Fig. 6 is an average value (n = 8–10) during this quasi-steady state, which is indicated by the linear increase in ln(XV), Fig. 5.

The effect of methanol concentration on α is presented in Fig. 6, where a critical specific growth rate μ_c = 0.0267 h^{-1} (at $S = S_1$ = 0.34 g/L) produced a maximum BoNT/A(H_c) per cell mass, α_m, of 1.72 mg BoNT/A(H_c)/g WCW. Using Eq. (9), ρ was calculated for each μ and is plotted in Fig. 6. The maximum specific product formation rate, ρ_m, is 0.082 mg/g WCW/h when $\mu = \mu_m$ = 0.0709 h^{-1} (at $S = S_2$ = 2.1 g/L). Imbalances of energy supply for growth and production is usually considered to be the reason that μ affects α or ρ (Chim-Anage et al., 1991; Shioya, 1992).

There are two strategies for optimizing BoNT/A(H_c) production depending on oxygen transfer capabilities. If the fermentation system has limited oxygen transfer capabilities the objective is to grow the cells to a pre-induction condition of $X = X_0$, $V = V_0$, where $J_0 = 0$ and $t = 0$. The culture is induced for as long as $\mu = \mu_m$ = 0.0709 h^{-1} (t_f >12 h) is maintained. Integration of Eq. (4) based on these conditions results in an expression for J_m.

$$J_m = \rho_m(X_0V_0)e^{\mu_m t_f}/\mu_m. \quad (26)$$

For the second case, the system is defined by the limitation of the media, i.e., final cell mass. The media describe in this work will support growth up to 450 g WCW/L. The objective is to optimize conditions so that once cell mass reaches a maximum that all of the cells contain the maximum amount of product. J_m is achieved when $\mu = \mu_c$:

$$J_m = \alpha_m X_f V_f. \quad (27)$$

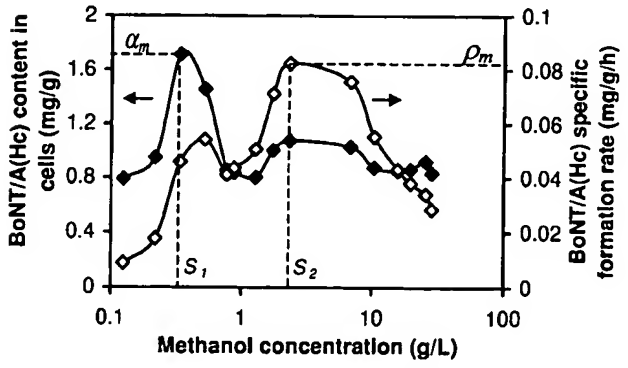

Figure 6. Effect of methanol concentration on BoNT/A(H_c) content in cells (♦) and specific formation rate (◇). S_1 = 0.34 g/L, S_2 = 2.1 g/L which correlate to a specific growth rate of μ_c = 0.0267, μ_m = 0.0709, respectively.

The constraints on the system for maximum production of BoNT/A(H_c) are an induction time of 12 h, final cell mass of 450 g/L and $\mu = \mu_c$ during induction. Using Eqs. (19) through (23), we developed a spreadsheet that models the entire fermentation process and calculates all of system parameters, such as length of glycerol fed-batch phase and induction phase on the basis of growth rates, starting and ending volume, and all media requirements. From the model, we determined that the maximum yield of BoNT/A(H_c) is 0.77 g/L with a total fermentation time of 54 h. This assumes the glycerol batch phase is 20 h (inoculum is 10% of the initial fermentation volume with an OD_{600} = 6) and that the fed-batch phase, which lasts 22 h, reaches a cell density of 410 g/L using a feed rate of 20 g/L/h (50% w/v glycerol) and results in a final cell mass of 450 g/L after a 12-h methanol induction at μ_c.

CONCLUSION

An unstructured growth model was developed for a Mut^+ strain of *P. pastoris* expressing BoNT/A(H_c). The model describes the relationships between the specific growth rate and methanol concentration and between the specific growth rate and specific methanol and ammonium consumption rates. The maximum specific growth rate (μ) calculated from the model was 0.08 h^{-1} at a methanol concentration of 3.65 g/L, while the realized maximum μ was 0.0709 h^{-1} and maximum specific methanol consumption rate was 0.0682 g/g WCW/h. When the effect of methanol concentration on specific growth rate was investigated, it was determined that above a methanol concentration of 3.65 g/L the cultured exhibited substrate inhibited and followed an uncompetitive inhibition model.

The results from this study showed that there is an optimum growth rate for optimum product formation of BoNT/A(H_c). It was determined that α_m reached a maximum of 1.72 mg/g wet cell mass when $\mu = \mu_c$ = 0.0267 h^{-1}, and ρ_m = 0.082 mg/g WCW/h when $\mu = \mu_m$ = 0.0709 h^{-1}. It is interesting to note that maximum intracellular product yield, α_m, was reached at approximately one-third of the maximum growth rate. At this time, the authors realize that the optimum growth rate for BoNT/A(H_c) formation is probably protein specific. Studies are in progress to test this model for other intracellular and secreted products.

NOMENCLATURE

BSM	basal salts medium
F	methanol feed rate (g/h)
J	total produced product (mg)
m	maintenance coefficient (g/g/h)
Q	total consumed amount of substrate (g)

S	methanol concentration (g/L)
S_{crit}	S to obtain μ_m' in Eq. (14) (g/L)
t	culture time (h)
V	broth volume (L)
V_{MeOH}	total consumed methanol (L)
V_{NH_3}	total consumed 28% ammonium (L)
WCW	wet cell weight (g)
X	cell density (g WCW/L)
$Y_{X/S}$	observed yield of biomass to substrate (g/g)
$Y_{X/S,t}$	true yield of biomass to substrate (g/g)

Greek Symbols

α	product content in cells (mg/g)
μ	specific growth rate (h^{-1})
μ_c	specific growth rate obtaining α_m
μ_m'	maximum μ in Eq. (14) (h^{-1})
ν	specific substrate consumption rate (g/g/h)
ρ	specific product formation rate (mg/g/h)

Subscripts

0	at initial time
f	at final time
m	maximum
MeOH	methanol
NH_3	28% ammonium
obs	observed
t	true

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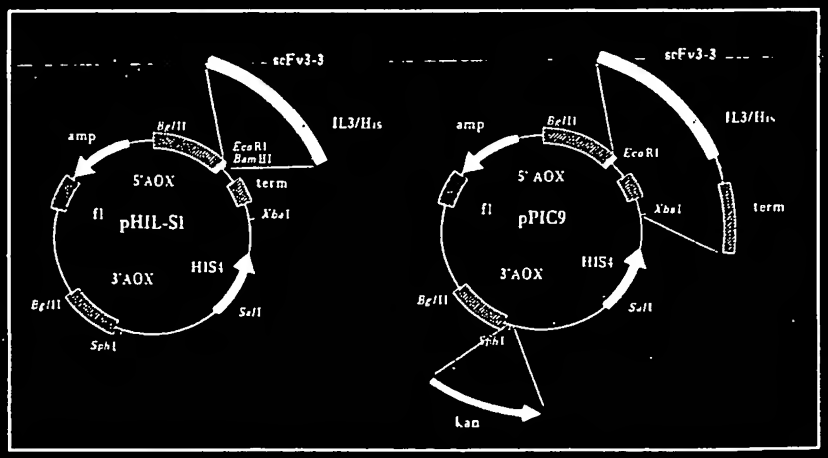

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esses, whether working with Mut⁺, Mut^S, or Mut⁻ phenotype strains, the strat
is to grow the yeast to high cell density on glycerol followed by induction
methanol. The three different phenotypes are distinguished by their growth
on methanol—Mut⁺ (0.14 h^{-1}), Mut^S (0.04 h^{-1}), and Mut⁻ (0.0 h^{-1})—and
induction procedures are described in this section.
temperature, pH, and DO should be set at 30°C, 5.0, and 35%, respectively
each should be set to automatic control. The air flow rate is set at 1.25 vvm
L) and is not changed during the fermentation.
initial glycerol concentration is 4% (w/v).
pH is controlled using a 30% by volume NH_4OH solution.
4-L fermentor (containing ~2 L of glycerol medium) is inoculated with the
250-mL shake flasks, each containing 50 mL of culture as described above.
initial glycerol batch phase usually lasts 20–24 h. Automatic DO control is
35% during the glycerol batch phase until the agitation rate reaches 800 rpm.
is point, the DO control is turned off and agitation is controlled manually
rpm for the 4-L fermentor; 450 rpm for the 60-L fermentor), adjusting the
of O_2 into the fermentor. When the glycerol is completely exhausted, the
eading will rise sharply. The OD_{600nm} at this point should be 60–70.
the glycerol fed-batch phase. The glycerol fed-batch phase further increases
ll density, initiates derepression of the *AOX1* promoter, and provides for a
h transition to the methanol fed-batch stage. The feed pump with glycerol
w/v) is set to deliver 15 mL/L h based on the starting volume of 2.0 L. For
ioFlo III fermentor, the nutrient feed pump is used to feed glycerol. This
will last for 4 h. During the glycerol fed-batch phase, the DO is controlled
lly by setting the agitation to maximum, the total air flow to constant, and
g the amount of oxygen to keep the DO steady at 30–35% (*see* **Note 4**).
g the glycerol fed-batch phase, perform "DO spikes" at 0.5, 1.0, 2.0, and
o ensure that glycerol is not accumulating. A DO spike consists of shutting
glycerol feed pump and timing how long it takes for the dissolved oxygen
g to rise 10%. Once the DO has increased 10%, the feed pump is turned
n. A final DO spike should also be performed at the end of the glycerol fed-
phase just prior to starting the methanol feed. The OD_{600nm} at this point of
mentation should be between 100 and 120.
ntifoam if necessary to control excess foaming. The BioFlo III is equipped
nsors and peristaltic pumps for automatic addition of antifoam. A solu-
ntaining 5% Struktol JA 673 antifoam (Struktol Company of America;
OH) in 100% methanol is used to control foaming. This solution can
added through a syringe filter. Undiluted antifoam (1–2 drops) may also
ed to the fermentor through a 0.45-μ syringe filter to control excessive
g.

hanol Feeding of Mut⁺ Strains

e two key points to the fed-batch strategy for Mut⁺ strains. The first
sure that all the glycerol is exhausted before beginning methanol

feeding, and the second is to make sure that methanol does not accumulate in the medium during the induction phase.

1. Turn off the glycerol feed pump. The DO should rapidly rise to 100% or greater.
2. Set the methanol feed rate at 3.5 mL/L·h. Adjust pH for optimum protein production (*see* **Note 5**). This low initial methanol feed rate allows the culture to adapt to the new carbon source. This period may last anywhere from 2 to 5 h. During adaptation, the DO will steadily decrease from 100 to 40% over a 1–3-h period. Eventually the DO will stabilize and then begin to increase. An increase in DO means that the culture is beginning to starve for methanol and is ready to accept a faster methanol feed rate. Perform a DO spike before changing either the methanol or O_2 feed rate (*see* **Note 6**). Once the DO reaches 60%, the methanol feed rate can be increased.
3. Increase the methanol feed rate to 5 mL/L·h. After 2 h, perform DO spike. Continue this feed rate until the DO spike time is 30 s or less. Once this occurs proceed to the next step.
4. Increase the methanol feed rate by 1 mL/L·h. After 1 h check the status of the culture by performing a DO spike. If the spike time is <30 s, increase the feed rate another 1 mL/L·h. If the spike time exceeds 35 s, maintain that feed rate until the DO spike time drops below 30 s.
5. Continue the incremental increases at ~1-h intervals until an optimal feed rate of 11–12 mL/L·h is achieved. If DO spike times become longer than 1 min, reduce the methanol feed rate by 1–2 mL/L·h. Ideally, the feed rate should be brought to the maximum as quickly as possible. The methanol induction period may be as short as 18 h or as long as 96 h. The best way to determine the optimum induction period is to run a fermentation for the maximum induction period, examining time-course samples throughout and empirically determining the optimum time of harvest. The harvest should take place at the point in time when the most product has accumulated. If you have the capability to measure methanol concentration, keep the concentration above 0.4% and below 3% ***(11)***. A residual methanol level of 1% provides the best growth conditions ***(11)***. The final OD_{600nm} may reach 400 or higher. After centrifugation of methanol-induced cultures, the cell pellet should occupy 30–40% of the culture volume.

3.2.3. Methanol Feeding of Mut^S Strains

The fermentation process for Mut^S strains is identical to Mut⁺ strains for steps described in **Subheading 3.2.1.** (glycerol batch and fed-batch steps). At the end of the glycerol fed-batch step, the methanol feed is started, and methanol concentration is maintained at <1% throughout the methanol-induction phase. Start the methanol feed rate at 1 mL/L·h and increase the rate over 6–8 h to 6 mL/L·h. Maintain that rate during the length of the induction period.

3.2.4. Methanol Induction of Mut⁻ Strains

The fermentation process for Mut⁻ expression strains is similar to that for *Escherichia coli* strains expressing proteins under control of the *lacZ* promoter.

3. DO spikes should be performed 15–30 min after initiating the glycerol feed and at 1–2 h intervals to ensure that the culture is limited for glycerol. DO spikes are performed by terminating the carbon feed and timing how long it takes for the DO to rise 10%, after which the carbon feed is resumed.

3.3.2.3. Methanol Fed-Batch Phase

1. Initiate by terminating the glycerol feed and starting a 100% methanol feed at a feed rate of approx 3.5 mL/h/L initial volume. The glycerol feed is left off for at least 5 min before proceeding with the methanol feed.
2. For low-pH fermentations, the pH of the fermentation medium can be decreased to 3.0 by changing the control set point to 3.0 and allowing the metabolic activity of the culture to lower the pH slowly to 3.0 over 4–5 h (*see* **Note 6**).
3. During the first 2–3 h, methanol will accumulate in the fermenter, and the dissolved oxygen will steadily decrease. DO spikes are not performed during this time. Increase agitation, aeration, pressure, or oxygen feeding during this phase to maintain the DO above 20%. If the DO cannot be maintained above 20%, stop the methanol feed, wait for the DO to spike, and continue on with the current methanol feed rate.
4. After the culture is fully adapted to methanol utilization (2–4 h) and the culture is limited on methanol, the feed rate is doubled to ~7 mL/h/L initial fermentation volume. This will be indicated by a steady DO reading and a fast DO spike time (generally under 1 min). It is recommended to maintain the lower methanol feed rate under limited conditions for 1 h before doubling the feed.
5. After increasing the feed rate to 7 mL/h/L, DO spikes should be performed after ~15 min and as often as necessary to ensure that the culture is limited on methanol (*see* **Note 7**).
6. After 2 h at the 7 mL/h/L feed rate, the methanol feed rate is again increased to approx 11 mL/h/L initial fermentation volume. This feed rate is maintained for a total of 24 h on methanol when the methanol feed can be further increased to 13 mL/h/L in order to ensure full methanol addition by harvest time.
7. The entire methanol fed-batch phase lasts ~70 h with a total of ~740 mL methanol fed/L of initial volume. For many recombinant proteins, a direct correlation between amount of methanol consumed and the amount of product produced has been observed. Therefore, some attention needs to be given to the total amount of methanol fed during the fermentation. The cell density continues to increase during the methanol fed-batch phase to a final level of ~450 ± 100 g/L wet cells. Because most of the fermentation is carried out in a fed-batch mode, the final fermentation volume will be approximately double the initial fermentation volume (*see* **Notes 8** and **9**).

3.3.2.4. Fermentation Results with Six-Copy Mut⁺ Strain

The results of the fermentation studies are summarized in **Table 2**. These fermentations were carried out in 2-L vessels starting out at a 1-L volume. As stated previously, the Mut⁺ six-copy strain was first tested under standard fer-

Table 2
Expression of IGF-I in *Pichia pastoris*: Fermentation Studies

Fermentation	Cassette copy #	Phenotype	pH	Western analysis, mg/L	Incstar RIA, mg/L	Nichols RIA, mg/L	C4 RP-HPLC, mg/L[a]	Cell density, wet g/L
A	6	Mut⁺	5	117	306	15	3	325
B	6	Mut⁺	3	555	1550	140	121	385
C	4	Mut⁺	3	nd[b]	1400	174	103	350
D	2	Mut⁺	3	nd	740	65	39	430
E	1	Mut⁺	3	21	167	35	14	415
F	6	Mut^s	3	nd	745	nd	nd	370

[a]HPLC values reported as authentic IGF-I values that represent ~20% of the total IGF-I forms present in HPLC.
[b]nd = not determined.

U.S. Patent No. 6,232,111, to Zhang *et al.*

US006232111B1

(12) **United States Patent**
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(45) **Date of Patent: *May 15, 2001**

(54) **METHOD FOR IMPROVING CULTURE MEDIUM FOR RECOMBINANT YEAST**

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(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(58) **Field of Search** 435/254.2, 254.21, 435/254.22, 254.23

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(57) **ABSTRACT**

Yields in yeast recombinant expression systems are improved by identifying bad lots and supplementing them with the appropriate combination of adenine, trehalose and/or lactate.

6 Claims, No Drawings

METHOD FOR IMPROVING CULTURE MEDIUM FOR RECOMBINANT YEAST

This application claims the benefit of U.S. Provisional Application No. 60/015,250, Apr. 10, 1996.

CROSS-REFERENCE TO RELATED APPLICATIONS

This is related to U.S. Ser. No. 086,216, filed Jul. 1, 1993, now published as WO 95/01422.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

Not applicable.

BACKGROUND OF THE INVENTION

Production of compounds of pharmaceutical significance by cultivation of recombinant yeasts is an expanding field of science and commerce. Purified recombinant hepatitis B surface antigen (HBsAg) is used as a vaccine for hepatitis B viral disease and is a well-known example of a pharmaceutically-significant recombinant protein.

Recombinant HBsAg is produced by cultivation of yeast cells in complex or chemically-defined (synthetic) culture media. Generally, complex media contain crude sources of nitrogen such as yeast extract and peptones. Although high yields of cells and crude HBsAg are achieved in these complex culture media, overall performance is frequently variable, and sometimes unacceptably inconsistent. Inconsistencies in fermentation performance adversely affect downstream purification steps and may also increase costs for the purified product.

Regulated expression systems are commonly used for the production of recombinant proteins. One type of regulated system provides tight nutritional control of the production of heterologous protein. This type of system maximizes biomass production and product stability while minimizing the adverse effects of heterologous protein expression on the host cell, e.g., Zabriskie et al., *Enzyme Microbial Technol.* 8:706–717 (1986).

For convenience, applicants employ a recombinant *S. cerevisiae* strain for the production of Recombivax HB® (a trademark of Merck & Co. Inc.), which strain harbors a plasmid composed of the coding sequence for HBsAg linked to the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, as well as an origin of replication from the yeast 2μ plasmid, and the LEU2 gene for selection in yeast cells. The strain is an adenine auxotroph, i.e., requires adenine for growth. Other adenine auxotrophs of yeast are typically used as recombinant hosts for heterologous protein expression, for example strains bearing mutations at the ADE 1 or ADE 2 loci. See, e.g., Kniskem, P. et al. in *Expression Systems for Processes for Recombinant DNA Products* (Hatch et al., eds.) ACS Symposium Series No.447 (ch.6) pp.65–75 (1991), and Schultz, L. et al. *Gene* 61, 123 (1987).

It would be desirable to identify the component(s) of complex media that affect fermentation performance, especially yields. Advantages of such discoveries would include a more reproducible fermentation process and a more predictable purification process.

Yeast extracts are commonly used in the media for yeast fermentations as the source for vitamins, trace elements and nitrogen nutrient. In many fermentation processes the nutrient which becomes limiting during the course of fermentation is the carbon source. The lot-to-lot variation of yeast extract due to variations in vendor's manufacturing processes dramatically affect recombinant yeast fermentation productivity and consistency, e.g. Recombivax HB® (a trademark of Merck & Co., Inc.) fermentation. The problem was partially solved in the past by the "brute-force" fermentation screening ("use-test") of new yeast extract lots. As a result, additional manpower and facilities had to be tied up, and sometimes "good" lots could not be secured due to delay in decision while other times "poor" lots were purchased and had to be thrown away. This disadvantage can be overcome by first identifying the critical and varying components in yeast extract that affect Recombivax HB® fermentation, and establishing rapid assay methods for these components. After a sufficiently representative database is built, the analytical results can be used to evaluate whether a particular yeast extract lot is desirable for Recombivax HB® fermentation.

The invention relates to a method to rapidly determine whether a yeast extract lot will be "good" for recombinant yeast fermentations, including that which produces HBsAg (Recombivax HB®), by measuring the contents of critical varying components such as adenine, trehalose and lactic acid. This simple and rapid screening procedure eliminates lots with suboptimal levels of these components and allows in most cases (about 80% of lots) superior and consistent fermentation productivity. The method also enables the improvement of fermentation yield by rational supplementation of those components to "poor" yeast extract lots.

Applicants have identified adenine and two metabolizable carbon sources (trehalose and lactate) as critical components in yeast extract causing fermentation inconsistency. Adenine is required for growth while the slowly metabolized trehalose supplies energy after growth phase for recombinant gene expression in the synthesis of expression product. The rapidly utilized lactate exerts a positive effect indirectly by sparing more ethanol as the carbon source for product synthesis. These effects on growth and production are mutually-dependent. A relatively high level of carbon sources (trehalose plus lactate, >4 g/42 g) and a mid level of adenine (0.06~0.1 g/42 g) are necessary characteristics of a good yeast extract lot for yeast cultivation and crude HBsAg production.

SUMMARY OF THE INVENTION

A method for improving the culture medium useful for the cultivation of recombinant yeasts and the production of recombinant proteins is provided. The medium is particularly useful for the cultivation of recombinant strains of *Saccharomyces cerevisiae* which produce HBsAg.

BRIEF DESCRIPTION OF THE DRAWINGS

Not applicable.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is related to a general fermentation process for the production of recombinant proteins by yeast cells. The process of the present invention is demonstrated

with the production of HBsAg by batch fermentation of strains of *Saccharomyces cerevisiae* transformed with a plasmid comprising the gene for HBsAg. As will be appreciated by one of ordinary skill in the art, the process of the present invention has a more general application to cultivation of other strains of *S. cerevisiae* and the production of other recombinant products and is not limited to HBsAg.

In general, yeast batch fermentation in complex medium is either a growth-limited process or a carbon source-limited process, depending on the adenine and trehalose/lactate contents of the YE (yeast extract) lot used. The concentration of these critical components in YE can vary dramatically due to variations in vendors' manufacturing processes. These inconsistencies contribute to fluctuations in fermentation performance, e.g., the amount of HBsAg produced. The analytical tools for adenine, trehalose and lactate in YE have been developed. Adenine content determines biomass production while carbon source (trehalose plus lactate) content affects antigen (HBsAg) product synthesis, and these two effects are related to each other. A mid-level adenine (0.06~0.1 g/42 g YE) and a high level trehalose plus lactate (>4 g/42 g YE) are the necessary requirements for a good lot, provided that the concentration of lactate does not exceed about 4.0 g/42 g YE. Concentrations of lactate exceeding about 4.0 g/42 g YE will cause significant change in fermentation pH profile. Many poor lots are improved by rational supplementation of adenine or trehalose or lactate or their combination.

In this invention, there is provided a method for improving culture medium with limiting carbon source for a recombinant yeast prototroph, comprising the steps of:

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentrations of trehalose and lactate;

c) adjusting the concentration of trehalose plus lactate to more than or equal to about 4.0 g/42 g of yeast extract, provided that the concentration of lactate is less than or equal to about 4.0 g/42 g yeast extract.

In one embodiment of this invention, there is provided a method for improving culture medium with limiting carbon source for a recombinant yeast prototroph, comprising the steps of:

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentrations of trehalose and lactate;

c) adjusting the concentration of trehalose plus lactate to between about 5.0 g/42 g of yeast extract and about 8.0 g/42 g of yeast extract, provided that the concentration of lactate is less than or equal to about 4.0 g/42 g yeast extract.

This invention also provides a method of identifying bad lots of yeast extract for fermentation with limiting carbon source for a recombinant yeast prototroph, comprising the steps of

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentrations of trehalose and lactate; and

c) identifying bad lots as those lots with suboptimal concentrations of trehalose or lactate.

In another embodiment of this invention, there is provided a method for improving culture medium with limiting carbon source for recombinant yeast adenine auxotrophs, comprising the steps of:

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentration of one or more of adenine, trehalose and lactate;

c) adjusting the concentrations of adenine to between about 0.06 to about 0.10 g/42 g of yeast extract, and of trehalose plus lactate to more than or equal to about 4.0 g/42 g of yeast extract, provided that the concentration of lactate is less than or equal to about 4.0 g/42 g yeast extract.

In another embodiment of this invention, there is provided a method for improving culture medium with limiting carbon source for recombinant yeast adenine auxotrophs, comprising the steps of:

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentration of one or more of adenine, trehalose and lactate;

c) adjusting the concentrations of adenine to between about 0.06 to about 0.10 g/42 g of yeast extract, and of trehalose plus lactate to between about 5.0 g/42 g of yeast extract and about 8.0 g/42 g of yeast extract, provided that the concentration of lactate is less than or equal to about 4.0 g/42 g yeast extract.

Another embodiment of this invention provides a method of identifying bad lots of yeast extract for recombinant yeast adenine auxotroph fermentation with limiting carbon source, comprising the steps of

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentration of one or more of adenine, trehalose and lactate; and

c) identifying bad lots as those lots with suboptimal concentrations of adenine, trehalose or lactate, or combination thereof.

Another embodiment of this invention is a method for improving culture medium with limiting carbon source for recombinant yeast adenine auxotrophs for the synthesis of recombinant Hepatitis B surface antigen, comprising the steps of:

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentration of one or more of adenine, trehalose and lactate;

c) adjusting the concentrations of adenine to between about 0.06 to about 0.1 g/42 g of yeast extract, and of trehalose plus lactate to more than or equal to about 4.0 g/42 g of yeast extract, provided that the concentration of lactate is less than or equal to about 4.0 g/42 g yeast extract.

Another embodiment of this invention is a method for improving culture medium with limiting carbon source for recombinant yeast adenine auxotrophs for the synthesis of recombinant Hepatitis B surface antigen, comprising the steps of:

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentration of one or more of adenine, trehalose and lactate;

c) adjusting the concentrations of adenine to between about 0.06 to about 0.1 g/42 g of yeast extract, and of trehalose plus lactate to between about 5.0 g/42 g of yeast extract and about 8.0 g/42 g of yeast extract, provided that the concentration of lactate is less than or equal to about 4.0 g/42 g yeast extract.

Another embodiment of this invention is a method of identifying bad lots of yeast extract for recombinant yeast

adenine auxotroph fermentation with limiting carbon source in the synthesis of recombinant Hepatitis B surface antigen, comprising the steps of

a) providing a quantity of a given lot of yeast extract to be tested;

b) measuring the concentration of adenine, trehalose and lactate; and

c) identifying bad lots as those lots with suboptimal concentrations of adenine, trehalose or lactate, or combination thereof.

It is understood that the yeast adenine auxotrophs are provided as illustrations of the techniques of identifying bad lots and rational supplementation of yeast extracts. Other yeast auxotrophs, as well as yeast prototrophs provide suitable sources for yeast extract analytical screening and supplementation for the purpose of synthesizing recombinant proteins.

In this invention, one preferred sum of the trehalose plus lactate content is more than or equal to about 4.0 g/42 g YE, provided that the concentration of lactate does not exceed about 4.0 g/42 g YE. This upper limit in lactate concentration avoids suboptimal yields from high fermentation pH. The concentration of trehalose is in principle unlimited, but at levels above about 8.0 g trehalose/42 g YE, it is typically not metabolized. At higher concentrations, no toxicity effect of trehalose has been observed. It is preferable to have at least both trehalose and lactate in the medium since they are providing an additional carbon source at different stages of fermentation. There are 42 g yeast extract (YE) per liter of the medium.

Improvement of Fermentation Performance of "Poor-Growth" Lots

It was observed that, in general, poor growth led to poor volumetric HBsAg (i.e. antigen) yield; yet abundant growth frequently also did not support good antigen production. Because the addition of ≧0.2 g/L adenine boosted growth to the range of that obtained with a "super-growth, poor-yield" lot, it was possible that the ample biomass production might have depleted other nutrients/factors related to and necessary for antigen synthesis. Therefore an adenine titration study was carried out using a "poor-growth" lot, which supported low antigen titer as expected. The results showed that while the growth increased progressively as the adenine concentration increased (up to 0.2 g/L), there was apparently an optimal level of adenine for antigen yield. In this case, adding 0.1 g/L led to a 60% increase in titer. The on-line respiration profiles of the cultures growing in another yeast extract lot clearly demonstrated that the original medium was limited in adenine and the addition of 0.04 g/L of adenine boosted growth dramatically. A 40% increase in biomass and 20% increase in antigen titer were achieved compared to the control batch. The sharp drop of OUR (Oxygen Uptake Rate) at ~32 hrs suggests that the higher growth supported by the higher adenine concentration quickly depleted ethanol (accumulated from glucose fermentation by the culture), a known provider of energy source for antigen synthesis, resulting in a smaller increase in antigen titer than biomass.

Enzymatic Assay for Adenine in YE

A method based on Naher (Methods of Enzymatic Analysis 4, 1909 (1974)) was developed, in which adenine is deaminated by nitrous acid to hypoxanthine, and oxidized by xanthine oxidase rapidly and qunatitatively to xanthine and further to uric acid measurable at 293 nm (see Examples). The conversion of adenine to uric acid during the assay was complete and quantitative. Finally, no formation of uric acid was observed when xanthine oxidase was omitted.

The adenine content measured for a YE lot was found to be insensitive to heat-sterilization conditions, indicating that adenine/growth relationship established at the 2-L shake-flask scale is applicable to large scale.

Relationship Between YE Adenine Content and Fermentation Performance

The biomass and antigen production of lots at the 2-L scale was measured as they relate to adenine content. Good correlation was obtained between growth and adenine in that biomass increased with adenine until the measured content reached about 0.12 g/42 g yeast extract(YE): after that the adenine level was no longer the limiting factor for growth. But no direct relationship between adenine and antigen yield existed except that most "good-yield" lots (≧38 mg HBsAg/L) possessed a mid-level of adenine (0.06–0.10 g /42 g YE), although some "poor-yield" lots were also found in this range. Thus, a mid-range adenine content is a desirable but not sufficient condition for optimal antigen (HBsAg) production.

Identification of Trehalose and Lactate as Metabolizable Carbon Sources in YE

Supplementation of adenine to some YE lots boosted growth but decreased HBsAg specific production, and some "supergrowth" lots due to high adenine contents supported very poor antigen yields. The likely explanation was that the abundant growth depleted the energy source such as ethanol required for antigen synthesis. On the other hand, a similar amount of ethanol should be produced from glucose (which is constant in every fermentation), yet in many cases growth or YE adenine content alone could not predict antigen yield of the fermentation, and drastically different yields were obtained for lots with very similar adenine or biomass level. A carbohydrate HPLC analysis was employed to examine YE components in conjunction with fermentation kinetic analysis. It was discovered that there were two metabolizable components in essentially every YE lot, a major disaccharide peak and a smaller "lactate" peak, and their levels varied lot-to-lot. Since these two peaks decreased or disappeared after fermentation, the corresponding compounds must have contributed to the fermentation by serving as carbon/energy sources.

The disaccharide peak was assigned as trehalose, an isomer of maltose, because treating the YE sample with a specific trehalase resulted in reduction of this peak and the formation of a glucose peak. As for the "lactate" component, incubation of the YE sample with L-lactate 2-monooxygenase led to a decrease in the peak size and the formation of an acetate peak. In order to confirm the structures of these two components, their purification from YE was carried out by hot ethanol extraction followed by preparative HPLC on an analytical column. The purified compounds were identified as trehalose and lactate by NMR studies.

Trehalose (α-D-glucopyranosyl α-D-glucopyanoside) is a storage material synthesized by baker's yeast in response to environmental stress. Trehalose content amounts up to 20% on dry cell weight basis. Since vendors' cultivation and downstream processes could not be absolutely consistent, trehalose content in various YE lots was found by HPLC to range widely from <1 to >7 g/42 g YE. As for the lactate component, since baker's yeast does not accumulate this metabolite, the minor amount detected (mostly <3 g/42 g YE) is often present due to lactobacillus contamination during the vendors'manufacturing processes, a common phenomenon in the baker's yeast industry.

The utilization of trehalose and lactate from YE during a yeast fermentation at the 23-L scale was monitored. It was

found that lactate was rapidly metabolized as carbon source for growth after glucose utilization, which delayed the depletion of the accumulated ethanol, a known energy source for antigen production. Broth pH increased during lactate utilization and dropped back down thereafter. Glycerol was accumulated but not re-utilized due to membrane impermeability. Trehalose was catabolized slowly during and after the oxidation of the accumulated ethanol, thus serving as carbon/energy source for the later phase of the fermentation during which recombinant product antigen (HBsAg) was being synthesized. Besides being an energy source, another plausible function of trehalose is the stabilization of cell membrane structure against environmental stress.

Relationship Between the Level of Trehalose Plus Lactate and Fermentation Performance

Various lots which had been evaluated in 2-L yeast fermentations were analyzed for their trehalose and lactate contents. The relationship between carbon source (trehalose plus lactate) contents and biomass gave no apparent correlation to relate growth and YE carbon source content, as most fermentations were limited by adenine. But there is a readily apparent trend that up to 6 g/42 g YE higher carbon source content supported higher antigen titers. The majority of the "good" lots (yielding >38 mg HBsAg/L) had >4 g/42 g YE in carbon source and lots with less than this level were essentially all "poor". However, not all the lots with respectable carbon source contents were "good". About 80% of the "good" lots possess mid-level adenine (0.06–0.1 g/42 g YE).

Effect of Lactate Supplementation on Fermentation Performance

There was a positive effect of lactate supplementation at 23-L scale to a YE lot containing high adenine (0.13 g/L) and low carbon sources (2.7 g tre, 0.6 g lact/L). Since lactate metabolism was found to increase pH, the pH was manually controlled to match the control. Clearly, the presence of 4.5 g/L more lactate provided carbon source for growth, thus sparing the ethanol. The resulting delay of ethanol depletion (as reflected by CO_2 Evolution Rate or CER) made more energy source available for antigen synthesis and hence led to higher HBsAg titer.

New Mechanism of Trehalose Effect and Improvement of Poor Lots by Rational Supplementation

One known function of trehalose is the protection of microbial membrane integrity against environmental stresses because of its unique characteristics in forming bonds with phosphodiester linkages in phospholipids. In the yeast fermentation, however, the positive effect of trehalose was often observed when trehalose was not intact, i.e., when it was split into glucose and catabolized. It appeared that trehalose affected yeast fermentation through slowly supplying glucose for growth and product synthesis. The later effect was major in that after ethanol depletion at 24–36 hrs (depending on the lot) which led to the cessation of exponential growth, trehalose became the sole carbon/energy source available for antigen synthesis, as the glycerol produced from glucose could not be re-utilized, and the lactate brought in by YE and Hy-soy had been depleted in earlier phase.

Based on such a new mechanism, a poor YE lot (high adenine, and low trehalose plus lactate content) is improved by providing additional trehalose. In one example, it was seen from the control that without additional trehalose, antigen synthesis essentially stopped when ethanol had depleted (judged by OUR) and most of the original trehalose was consumed at ~30 hrs. Addition of more glucose at 0 hr resulted in accumulation of more ethanol (and more non-usable glycerol) for growth, which slightly delayed the depletion of ethanol, and thus could only slightly increase antigen titer. When trehalose was supplemented to the level of about 8 g/42 g YE, similar catabolic profiles were observed, and trehalose utilization provided carbon/energy during synthesis phase which led to more active cells (as reflected by OUR profiles) and significantly higher antigen yield. It is noteworthy that more trehalose did not delay ethanol depletion as seen with more glucose, indicating different mechanisms and the importance of the slowly-released carbon/energy source which ensured the availability of energy for antigen synthesis.

The effect of trehalose supplementation to various low-to mid-trehalose lots at 23-L fermentor scale indicated that most of them were improved mainly through the increase in specific production, while the biomass was increased only slightly compared to antigen titer. In most cases the on-line OUR profiles showed the distinctive higher respiratory activities at the synthesis phase compared to the respective controls.

EXAMPLE 1

Culture Inoculum Development and Production Fermentation

The culture source for all the experiments was frozen seed stocks, generated from frozen vials of *Saccharamyces cerevisiae* 2150-2–3 (pHBS56-GAP347/33).

The medium for all seed stages was 5×Leu$^-$ containing 90 g/L dextrose. The production fermentation medium was Enhanced YEHD, comprised of 42 g/L yeast extract (YE), 35 g/L Hy-Soy peptone and 17 g/L dextrose (sterilized separately), with the presterilization pH adjusted to 5.0. Polyalkylene glycol was added as antifoam at 0.5 ml/L for shake-flask fermentation and 1 ml/L for stirred-tank fermentation. Adenine, lactate or trehalose was added prior to sterilization, at the concentrations specified.

A frozen cell suspension (1.5 ml) was thawed at room temperature and inoculated to a 250-mL Erlenmeyer flask containing 50 ml of medium. After 24 h incubation on a rotary shaker (220 rpm. 28° C.), twenty ml of the culture were transferred to a 2-L Erlenmeyer flask containing 500 ml of medium, and cultivated for 24 -h on a rotary shaker at 180 rpm and 28° C. The culture was used as the inoculum for fermentation studies in the 2-L shake-flasks and in some 23-L tanks. For other 23-L scale fermentations, a third seed stage was included which was developed for 24 h in a 23-L tank containing 15 liters of medium, at 28° C. with an agitation of 600 rpm and aeration of 6 L/min.

For fermentation studies carried out at shake-flask scale, the 2-L baffled flask containing 200 ml of Enhanced YEHD medium was used. The flasks were inoculated with 4% (v/v) seed culture and incubated at 28° C. and 180 rpm on a rotary shaker for two days. For 23-L stirred-tank fermentations, an inoculum of 5% from the shake flask seed or 8% from the third stage seed was used. The tanks were operated at 28° C. with an agitation of 600 rpm, an aeration of 12 L/min, and a back pressure of 0.6 bar. Respiratory activities (Oxygen Uptake Rate or OUR, and CO_2 Evolution Rate or CER), dissolved oxygen and pH were monitored on-line, while carbohydrates were monitored off line by HPLC.

EXAMPLE 2

Analysis

Growth was measured by optical density (OD) at 660 nm on a spectrophotometer, or by dry cell weight (DCW). These two methods gave essentially the same conclusions. Carbon source compounds such as glucose, trehalose, lactate and ethanol were analyzed by HPLC system. To profile antigen

production, cell pellets of 50 OD units were prepared from fermentation broth samples taken at various time points, washed once with PBS buffer and stored at −70° C. till breakage. The lysate was prepared by vortexing the cells with glass beads. The protein content in cell lysates was analyzed by the bicinchoninic acid method, and the HBsAg concentration was determined by enzyme immunoassay (EIA) using the commercially available assay kit. All results were back-calculated and expressed as fermentation titers (mg/L).

The data was based on the assays carried out at the same time and under the same conditions for the experimentals and the respective controls to minimize variations from assay kits, standards, and assay conditions. Similarly, all the comparisons were based on the same experiment to eliminate differences due to culture conditions. When two or more measurements were carried out, average results were used.

EXAMPLE 3

Measurement of Adenine Content in Yeast Extracts

Adenine content in various YE lots was determined by an enzymatic assay developed based on Naher (Methods of Enzymatic Analysis 4, 1909 (1974)) which involves adenine deamination by nitrous acid and oxidation by xanthine oxidase to give uric acid measurable at 293 nm. The procedure is as follows:

1. Prepare 42 g/L YE sample by adding 24.5 ml of water and 0.2 ml of 2 N HCl to 1.05 g YE powder and mixing throughly to get clear solution (the lot giving turbid solution is not desirable). Also prepare adenine standard solutions (0, 0.025, 0.05, 0.10, 0.20, 0.40 g/L) by diluting with water a 1.0 g/L, pH 2 stock solution (stable at 4° C. for months).
2. Mix throughly by vortexing 2.0 ml of the YE sample or the adenine standard with 0.9 ml of 20% (w/v) sodium nitrate and 0.1 ml of undiluted sulfuric acid in a 50-mL uncapped tube. Immediately put the mixture into a 37° C. water bath to incubate for 60 min with paper towel covering the uncapped tube.
3. After taking out the tube add 1.0 ml of 20% (w/v) sodium hydroxide solution and mix well to stop reaction. This mixture serves as the assay solution in the following steps and is found stable at 4° C. for at least a month.
4. Saturate Tris buffer (0.1 M, pH 8.0) with oxygen by sparging air to the buffer. Add 3.0 ml of this buffer and 30 μl of the assay solution to a 5-mL cuvette. Seal the cuvette with parafilm and invert to mix the content, and immediately read the extinction (E1) at 293 nm on a spectrophotometer blanked with the standard containing 0 g/L adenine. Two readings should be made for each measurement and the values should not differ more than 0.002.
5. Add 10μl of 1:10-diluted xanthine oxidase suspension (15.61 U/ml, diluted with 3.2 M ammonium sulfate) to the cuvette and seal the cuvette with parafilm. Invert to mix the content, and read the extinction at 292 nm the same way as above on the same spectrophotometer immediately and then every 5 min until a constant/maximal value (E2) is reached (generally in less than 30 min).
6. Adenine concentration in a YE lot (g/42 g YE) is estimated from its E value based on a standard curve generated from the authentic adenine samples (0, 0.025, 0.05, 0.10, 0.20, 0.40 g/L, treated the same way and at the same time as the YE samples). E is calculated according to the following equation ("blank" has 0 g/L of adenine):

$$E=(E2-E1)\text{sample}-(E2-E1)\text{blank}$$

EXAMPLE 4

Measurement of Trehalose and Lactate

Trehalose and lactate contents in various yeast extract (YE) lots were determined by HPLC method using an ion-exchange column. The procedure is as follows:

1. Prepare 42 g/L YE sample the same way as that for adenine analysis. Dilute the sample (1:5) with 0.005 M sulfuric acid (mobile phase) before filtering through a 0.45μ membrane. Also prepare trehalose (as dihydrate) standard solutions (0–2.0 g/L) and Na-lactate standard solutions (0–1.0 g/L) with the mobile phase.
2. Generate the standard curves for trehalose and lactate on an HPLC system, and then analyze the YE sample. The equipment includes a solvent delivery pump, an automatic sampler injector and a detector. A 20-μl sample is injected into column containing a polystyrene divinylbenzene cation exchange resin (for organic acids and alcohols) maintained at 60° C. The sample is eluted isocratically with 0.005 M sulfuric acid at 0.7 ml/min, and monitored for refractive index (RI) change. Sample peaks are identified and quantified by comparing with those of authentic compounds. Under these conditions, trehalose eluted at ~7.3 min and lactate at ~12.6 min.

EXAMPLE 5

Purification of Trehalose and Lactate

Purification of trehalose and lactate from YE in order to confirm the structures by NMR was achieved through hot ethanol extraction followed by preparative HPLC on an analytical column. To 50 g of YE was added 200 ml of ethanol and the mixture was stirred for 30 min in an 85–90° C. water bath. The filtrate was allowed to cool at room temperature and the resulted precipitate was collected. After washing with cold ethanol and dried with air, the precipitate was dissolved in 2 ml of water. The preparation, estimated to be ≧30% in weight purity in terms of trehalose, was injected and eluted repeatedly on the above analytical HPLC system for further purification (no prep column was available). The pooled trehalose and lactate fractions were dried by lyophilization before NMR structure determination.

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention emcompasses all of the usual variations, adaptations, or modifications, as come within the scope of the following claims and its equivalents.

What is claimed:

1. A method of producing a yeast extract composition for use as an additive to culture media having a limiting carbon source concentration for a recombinant *Saccharomyces cerevisiae* prototroph, comprising the steps of:

a) providing a quantity of yeast extract to be tested;

b) measuring the concentrations of trehalose and lactate in said quantity of yeast extract;

c) adjusting the concentration of trehalose plus lactate to more than or equal to 4.0 g per 42 g dry weight of yeast extract if the concentration of trehalose plus lactate in the yeast extract as measured in step b) is less than 4.0 g per 42 g dry weight of yeast extract;

to produce a yeast extract composition for use as an additive to culture media having a limiting carbon source concentration for a recombinant *Saccharomyces cerevisiae* prototroph, wherein the yeast extract composition obtained has a concentration of trehalose plus lactate of more than or equal to 4.0 g per 42 g dry weight of yeast extract.

2. The method according to claim 1, wherein the adjustment in the concentration of trehalose plus lactate according to step c) is between about 5.0 g/42 g and about 8.0 g/42 g.

3. A method of producing a yeast extract composition for use as an additive to culture media having a limiting carbon source concentration for a recombinant *Saccharomyces cerevisiae* adenine auxotrophs, comprising the steps of:

a) providing a quantity of yeast extract to be tested;

b) measuring the concentrations of adenine, trehalose and lactate in said quantity of yeast extract;

c) adjusting the concentrations of adenine to between about 0.06/42 g to about 0.10 g/42 g dry weight of yeast extract if the concentration of adenine measured in step b) is less than 0.06 g/42 g dry weight, and adjusting the concentration of trehalose plus lactate to more than or equal to about 4.0 g/42 g dry weight of yeast extract if the concentration of trehalose plus lactate measured in step b) is less than 4.0 g/42 g dry weight,

to produce, a yeast extract composition having a concentration of adenine between about 0.06 g/42 g to about 0.10 g/42 g dry weight of yeast extract, a concentration of trehalose plus lactate of more than or equal to 4.0 g per 42 g dry weight of yeast extract and a concentration of lactate of less than or equal to about 4.0 g/42 g dry weight of yeast extract.

4. The method according to claim 3, wherein the adjustment in the concentration of trehalose plus lactate according to step c) is between about 5.0 g/42 g and about 8.0 g/42 g.

5. A method of producing a yeast extract composition for use as an additive to culture media with limiting carbon source concentration for recombinant *Saccharomyces cerevisiae* adenine auxotrophs for the synthesis of recombinant Hepatitis B surface antigen, comprising the steps of;

a) providing a quantity of yeast extract to be tested;

b) measuring the concentrations of adenine, trehalose and lactate in said quantity of yeast extract;

c) adjusting the concentrations of adenine to between about 0.06/42 g to about 0.10 g/42 g dry weight of yeast extract if the concentration of adenine measured in step b) is less than 0.06 g/42 g dry weight, and adjusting the concentration of trehalose plus lactate to more than or equal to about 4.0 g/42 g dry weight of yeast extract if the concentration of trehalose plus lactate measured in step b) is less than 4.0 g/42 g dry weight,

to produce a yeast extract composition having a concentration of adenine between about 0.06/42 g to about 0.10 g/42 g dry weight of yeast extract, a concentration of trehalose plus lactate of more than or equal to 4.0 g per 42 g dry weight of yeast extract and a concentration of lactate of less than or equal to about 4.0 g/42 g dry weight of yeast extract.

6. The method according to claim 5, wherein the adjustment in the concentration of trehalose plus lactate according to step c) is between about 5.0 g/42 g and about 8.0 g/42 g.

* * * * *

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High-yield Secretion of Recombinant Gelatins by *Pichia pastoris*

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Recombinant non-hydroxylated gelatins based on mouse type I and rat type III collagen sequences were secreted from the methylotrophic yeast *Pichia pastoris*, using the *Saccharomyces cerevisiae* α-mating factor prepro signal. Proteolytic degradation could be minimized to a large extent by performing fermentations at pH 3·0 and by adding casamino acids to the medium, even though gelatin is extremely susceptible to proteolysis due to its open, unfolded structure. Proteolytic cleavage at specific mono-arginylic sites, by a putative Kex2-like protease, could be successfully abolished by site-directed mutagenesis of these sites. Production levels as high as 14·8 g/l clarified both were obtained, using multicopy tranformants. To our knowledge, this represents the highest level of heterologous protein secretion reported to date for *P. pastoris*. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — *Pichia pastoris*; gelatin; collagen; expression; secretion; proteolysis; Kex2

INTRODUCTION

Gelatin is a well-known biopolymer and has a long history of use, mainly as a gelling agent in food. It is, in essence, denatured collagen and is prepared by hot acid or alkaline extraction of animal tissues such as bones and hides (Asghar and Henrickson, 1982). The gelling properties of gelatin are due to the remarkable amino acid sequence of its collagen ancestor. The so-called helical domain of this sequence consists of repeating Gly–Xaa–Yaa triplets, where Xaa and Yaa are often proline and hydroxyproline, respectively. *In vivo*, this polymeric structure enables the assembly of three procollagen molecules into a collagen triple helix (Vuorio and De Crombrugghe, 1990). This is a highly ordered process by virtue of specific mutual recognition of the collagen propeptides. In contrast, the denatured collagen triple helices in a heated gelatin preparation renature upon cooling to form randomly intertwining triple helices, resulting in the characteristic gel formation.

Apart from its traditional use, gelatin is also employed in sophisticated technical and medical applications, such as intravenous infusions (Saddler and Horsey, 1987), matrix implants (Pollack, 1990) and injectable drug delivery microspheres (Rao, 1995). The suitability of traditional gelatin for certain applications is, however, limited by its inherent characteristics. First, the above-mentioned extraction procedure results in chemically modified gelatin peptides covering a wide range of molecular weights (Ashgar and Henrickson, 1982). Second, traditional gelatin has a high gelling temperature due to its high content of helix-stabilizing hydroxyprolines. This can be undesirable for low temperature applications (Saddler and Horsey, 1987).

In principle, gelatins of specific molecular weight can be prepared by biotechnological means. Production of non-gelling (*i.e.* non-hydroxylated) gelatin-like proteins in *Escherichia coli* has been reported. It involves the expression of synthetic genes constructed from repeating $(Gly–Xaa–Yaa)_n$-encoding units (Goldberg *et al.*, 1989; Cappello, 1990; Obrecht *et al.*, 1991; Gardner *et al.*, 1992). Many problems arise concerning the instability of these highly repetitive genes (Cappello, 1990). Probably, native gelatin sequences derived from collagen genes are more stable than synthetic gelatin-like sequences, because there is greater variation in amino acid

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usage and less repetitiveness. Production in yeasts rather than bacteria may be preferable, since it has been reported that yeasts are well able to cope with repetitive genes (Strausberg and Link, 1990; Cappello and Ferrari, 1994; Fahnestock and Bedzyk, 1997). Furthermore, the methylotrophic yeasts especially can produce high levels of recombinant protein (Hollenberg and Gellissen, 1997). It has recently been shown that proline hydroxylation can be achieved in the yeasts *P. pastoris* and *S. cerevisiae* by co-expression of heterologous prolyl-4-hydroxylase (Vuorela *et al.*, 1997; Vaughan *et al.*, 1998). Although we currently pursue the production of non-gelling gelatins, yeasts may thus also offer the possibility to produce hydroxylated, gelling gelatins. Whichever host organism is chosen, a challenge remains to overcome the extreme susceptibility of non-hydroxylated gelatin to proteolytic degradation, resulting from its random coil conformation. In this paper we report the high yield extracellular production of native non-gelling gelatins by the methylotrophic yeast *P. pastoris* and we describe the minimization of their proteolytic degradation.

MATERIALS AND METHODS

Yeast strains and plasmids

P. pastoris strains GS115 (*his4*; Cregg *et al.*, 1985) and SMD1168 (*his4, pep4*) and expression vector pPIC9 (Clare *et al.*, 1991a) were obtained from Invitrogen. The pPIC9 plasmid contains a *HIS4* selectable marker, an alcohol oxidase 1 (*AOX1*) promoter/terminator cassette and a *S. cerevisiae* α-factor prepro secretory signal. Vector pPIC9K was obtained from M. A. Romanos. It is identical to pPIC9 except that it also contains the *Tn903 kan*^{r} gene, which enables the selection of multicopy transformants (Scorer *et al.*, 1994).

Media composition

Minimal dextrose plates consisted of 0·4 mg/l biotin, 1·34% Yeast Nitrogen Base without amino acids (Difco), 1% dextrose and 1·5% agar. Minimal glycerol medium contained 0·4 mg/l biotin, 1·34% Yeast Nitrogen Base without amino acids and 1% glycerol. Buffered minimal glycerol medium consisted of minimal glycerol medium supplemented with 100 mM potassium phosphate, pH 6·0. Buffered minimal methanol medium was identical to buffered minimal glycerol medium but contained 0·5% methanol instead of glycerol. Fermentation basal salts medium contained, per litre: 26·7 ml phosphoric acid (85%), 0·93 g calcium sulphate dihydrate, 18·2 g potassium sulphate, 14·9 g magnesium sulphate heptahydrate, 4·13 g potassium hydroxide, 40·0 g glycerol and 4·3 ml trace elements. Trace elements contained, per litre: 4·5 g cupric chloride dihydrate, 0·09 g potassium iodide, 3·5 g manganese chloride tetrahydrate, 0·2 g sodium molybdate dihydrate, 0·02 g boric acid, 1·08 g cobalt sulphate heptahydrate, 42·3 g zinc sulphate heptahydrate, 65·0 g ferrous sulphate heptahydrate, 0·2 g biotin and 5·0 ml sulphuric acid.

Construction of pCOL3A1 and pCOL3A1K expression vectors

Plasmid pRGR5, containing a partial rat proα1(III) collagen cDNA (Glumoff *et al.*, 1994; Genbank Accession No. X70369), was digested with *Pst*I, yielding a 0·7 kb fragment encoding part of the helical domain. The resulting fragment was blunt-ended and inserted into the *Sna*BI site of pPIC9, yielding vector pCOL3A1. The vector pCOL3A1K was constructed by inserting a 1·0 kb *Bam*HI/*Eco*RI fragment isolated from pCOL3A1 into the *Bam*HI/*Eco*RI digested vector pPIC9K.

Construction of pCOL1A1-1 and pCOL1A1-2 expression vectors

Two mouse type I collagen encoding DNA fragments of different length were amplified by PCR, such that both fragments shared the same 5′ end. The primers were based on the mouse *COL1A1* sequence published by Li *et al.* (1994; Genbank Accession No. U08020) and were as follows: C1A1-FW: 5′-CTTCCCAGATGTCCTA TGGCTATGATG-3′, C1A1-RV1: 5′-CCGCTC GAGGCGCTCGCCAGGAGGTCCAGGCAG-3′ and C1A1-RV2: 5′-GCGCTCGAGGGGAGGA CCAATGGGACCAGTCAG-3′. PCR was performed on Mouse 17-day Embryo QUICK-Clone® cDNA (Clontech), using Advantage® KlenTaq Polymerase Mix (Clontech). A 1·0 kb product (COL1A1-1) was obtained using primer combination C1A1-FW/C1A1-RV1 and a 1·8 kb product (COL1A1-2) was obtained using primer combination C1A1-FW/C1A1-RV2. To enable cloning of these fragments, the pPIC9 vector was modified by insertion of an adapter, which was prepared by annealing two complementary oligonucleotides: 5′-TCGAAAAGAGAGAGGCTGA AGCTCCCATGGGATAACTCGAGTAGG-3′

and 5′-AATTCCTACTCGAGTTATCCCATGG GAGCTTCAGCCTCTCTCTTT-3′. This adapter was inserted into the *Xho*I/*Eco*RI sites of pPIC9, whereby the original *Xho*I site was disrupted. The COL1A1-1 and COL1A1-2 PCR products were digested with *Nco*I/*Xho*I, after which the resulting helical domain encoding DNA fragments were cloned into the *Nco*I/*Xho*I sites within the adapter. The vectors pCOL1A1-1 and pCOL1A1-2 thus obtained were verified by DNA sequencing using an ALF automated sequencer (Pharmacia).

Construction of pCOL1A1-1 and pCOL1A1-2* expression vectors*

Using plasmid pCOL1A1-1 as a template and using *Pwo* DNA polymerase (Boehringer–Mannheim), PCR was performed using the commercial vector primers 5′-AOX1 and 3′-AOX1 (Invitrogen) in combination with the following mutagenic primers (mutagenic positions are underlined): MUT1FW: 5′-GAGCCTGGCGGT TCAGGTCCACGAGGTCCAATGGGTCCCC CTGG-3′, MUT1RV: 5′-CCAGGGGGACCCA TTGGACCTCGTGGACCTGAACCGCCAGG CTC-3′, MUT2FW: 5′-GGAGCTCCTGGCCA GCGAGGTCCAATGGGTCTGCCCGGTGAG AG-3′, MUT2RV: 5′-CTCTCACCGGGCAGAC CCATTGGACCTCGCTGGCCAGGAGCTCC-3′. The primer combinations used were 5′-AOX1/ MUT1RV, MUT1FW/MUT2RV and MUT2FW/3′-AOX1. The 0·5 kb, 0·3 kb and 0·7 kb PCR products obtained, respectively, were combined by overlap extension PCR (Ho *et al.*, 1989) to form a doubly mutated 1·5 kb product. Digestion with *Bam*HI/*Apa*I resulted in a 1·0 kb DNA fragment which was subsequently inserted into *Bam*HI/*Apa*I-digested pCOL1A1-1 and pCOL1A1-2. The plasmids pCOL1A1-1* and pCOL1A1-2* thus obtained were verified by sequencing.

Transformation of P. pastoris

Plasmids used for transformation were linearized with *Sal*I in order to preferentially obtain Mut$^+$ transformants (*i.e.* by integration at the *his4* locus and thus enabling normal growth on methanol) or with *Bgl*II in order to preferentially obtain Mut^S transformants (*i.e.* by integration at the *AOX1* locus and thus resulting in slow growth on methanol; Clare *et al.*, 1991b). *P. pastoris* was transformed by electroporation (Becker and Guarente, 1991) using a GenePulser (Biorad) set at 1500 V, 25 μF and 200 Ω and using 0·2 cm cuvettes. After growth on minimal dextrose plates at 30°C for 3 days, several colonies were selected for PCR confirmation of the Mut genotype according to Linder *et al.* (1996), with the exception that extension at 72°C was for 2 min and cells were used directly for PCR without any pretreatment.

Selection of multicopy P. pastoris *transformants*

Multicopy Mut$^+$ pCOL3A1K transformants were selected on basis of G418 resistance, essentially as described by Scorer *et al.* (1994). Copy numbers were estimated by a quantitative dot blot, as described by Clare *et al.* (1991b), with the following modifications. The blot was hybridized to a *COL3A1* probe. A *URA3* probe was used for normalization of the amounts of DNA transferred. The blots were subjected to autoradiography and the signals were quantified by using a densitometric scanner (PDI).

Small-scale production of recombinant gelatins in P. pastoris

Transformants were grown overnight in buffered minimal glycerol medium, harvested and resuspended in buffered minimal methanol medium to an OD_{600} of 1·0 (Mut$^+$) or 10·0 (Mut^S). Cells were grown in baffled shaking flasks for 4 days at 30°C and 250 rpm, with methanol being added to 0·5% every day.

Fermentative production of recombinant gelatins in P. pastoris

Fermentations were performed in 1 l, 2 l, 20 l or 140 l fermenters (Applikon). At the start of the fermentation, the fermenter contained half the working volume of fermentation basal salt medium, which optionally contained 1% casamino acids. Fermentation conditions for Mut$^+$ transformants were as follows. The temperature was set at 30°C, agitation at 500 rpm and aeration rate at 1 vvm. The pH was adjusted to pH 5·0 with 25% ammonium hydroxide. The fermenter was inoculated with 10% of the initial fermentation volume of a culture grown in minimal glycerol medium. A batch culture was grown until the glycerol was completely consumed. Aeration and agitation were increased to 2 vvm and 1000 rpm, respectively. A glycerol fed-batch phase was initiated by feeding 50% glycerol containing 12 ml/l trace salts, at a rate of 18 ml/h per litre initial fermentation volume. The pH was either maintained at 5·0 by the

addition of 25% ammonium hydroxide, or was allowed to drop to 3·0 by the yeast's metabolism. The glycerol feed was discontinued when the cell wet-weight reached approximately 180 g/l. After complete consumption of the glycerol, additional casamino acids were optionally added to 1% of the initial fermenter volume and the culture was induced by initiating a 100% methanol feed containing 12 ml/l trace salts. The feed rate was initially set at 3 ml/h per litre initial fermenter volume, and was gradually increased to maximally 9 ml/h per litre initial fermenter volume. Fermentation conditions for Mut^S transformants were similar, but the methanol feed rate was initiated at 1 ml/h per litre initial fermenter volume and was gradually increased to 3 ml/h per litre. Throughout the fermentation, 2 ml culture samples were collected and spun at 20 000 × *g* for 5 min, after which the wet cell pellets were weighed. The supernatants were filtered using a disposable 0·22 µm filter.

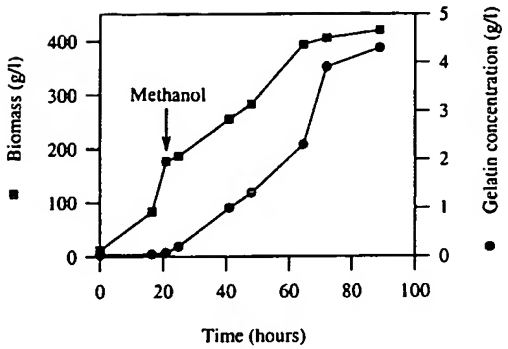

Figure 1. Growth and productivity curves of a 20 l COL3A1 fermentation at pH 3·0. The culture was grown in glycerol batch mode for 0–16·5 h and in glycerol fed-batch mode for 16·5–21 h, after which protein expression was induced by initiating a methanol-fed batch. Biomass is expressed as cell wet-weight (g) per litre of broth. Product concentrations are expressed in gelatin (g) per litre of clarified broth and were determined using the BCA Protein Assay (Pierce) with analytical grade gelatin as a reference, after removal of endogenous secreted proteins by acetone fractionation (see Materials and Methods).

Quantification of produced gelatins

In order to quantify the amounts of gelatin secreted into the medium, the gelatin was separated from extracellular endogenous proteins by acetone fractionation. Endogenous proteins were precipitated at 40% (v/v) acetone. The gelatin in the supernatant was precipitated by further increasing the acetone concentration to 80% (v/v), after which the resulting pellet was washed with 80% (v/v) acetone. The effectiveness of this separation was demonstrated by SDS–PAGE and by the observation that the determined amino acid composition closely matched that of pure gelatin (most markedly the approximate 33% Gly and 22% Pro). Gelatin concentrations were subsequently determined using the enhanced protocol of the BCA Protein Assay (Pierce) with analytical grade gelatin (Merck) as a reference.

SDS–PAGE and N-terminal protein sequencing

SDS–PAGE (Laemmli, 1970) was performed in a Mini-PROTEAN II system (Biorad) under reducing denaturing conditions. For N-terminal protein sequencing, proteins were blotted onto Immobilon P^{SQ} membrane (Millipore) by applying 100 V for 1 h in a Mini Trans-Blot Cell (Biorad). Transfer buffer was 2·2 g CAPS per litre of 10% methanol, pH 11. Blots were stained with Coomassie brilliant blue and selected bands were cut out. N-terminal protein sequencing using Edman degradation was performed by Sequentiecentrum Utrecht, The Netherlands.

RESULTS

Production of Col3a1 gelatin

A 0·7 kb rat *COL3A1* cDNA fragment, encoding a 21 kDa gelatin, was cloned into the *P. pastoris* expression vector pPIC9. The vector pCOL3A1 thus obtained was used to transform *P. pastoris* GS115. For each construct, several Mut^+ transformants were randomly chosen and tested for gelatin production in shaking flasks. SDS–PAGE of the culture supernatants showed no significant differences in productivity between different transformants.

A representative COL3A1 transformant was selected for fermentation experiments. A growth and productivity curve of a typical COL3A1 fermentation of pH 3·0 is shown in Figure 1. Routine Col3a1 production levels of 4–5 g/l clarified broth were produced at a biomass wet-weight of 300–500 g/l.

Culture supernatants harvested throughout the fermentation were subjected to SDS–PAGE. Collagenous proteins are known to migrate in SDS–PAGE at an apparent molecular weight approximately 1·4 times higher than the true

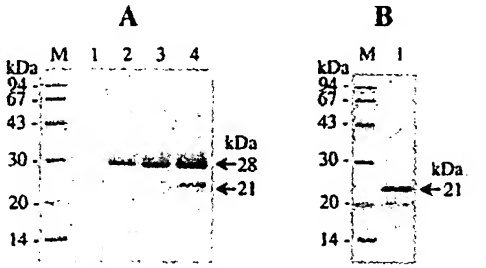

Figure 2. SDS–PAGE of COL3A1 fermentations at pH 3·0 and pH 5·0. (A) Lanes 1–4: 2 µl culture supernatant of a fermentation at pH 3·0, collected at 16·5, 41, 64·5 and 89 h, respectively. Lane M: molecular weight marker (Pharmacia). (B) Lane 1: 2 µl culture supernatant of a fermentation at pH 5·0, collected at 93 h. Lane M: molecular weight marker.

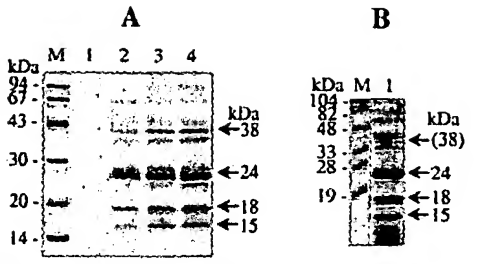

Figure 3. SDS–PAGE of COL1A1 fermentations at pH 3·0 with or without supplementation of the media with casamino acids. (A) Lanes 1–4: 3 µl culture supernatant of a fermentation supplemented with casamino acids, collected at 21, 45, 70 and 94 h, respectively. Lane M: molecular weight marker. (B) Lane 1: 10 µl culture supernatant of a fermentation without supplementation with casamino acids, harvested at 94 h. Note that while three times more supernatant was loaded than in lane 4 of (A), the full-length 38 kDa band can barely be discerned from background proteins. Lane M: prestained molecular weight marker (Biorad).

molecular weight (Butkowski *et al.*, 1982). Accordingly, the apparent molecular weight observed for the 21 kDa Col3a1 gelatin was 29 kDa (Figure 2A). Apart from the full-length band, minor degradation products of about 21 kDa were visible. Initially, fermentations were performed at pH 5·0, but during the course of fermentation the full-length band was completely degraded into 21 kDa and smaller bands (Figure 2B). This pH effect suggests that the proteases involved were extracellular neutral proteases. It has been reported that supplementation of the fermentation medium with casamino acids can reduce extracellular proteolysis (Clare, 1991a). When applied to Col3a1 fermentations at pH 3·0, no such effect was observed (data not shown).

Circular dichroic spectroscopy performed according to De Wolf and Keller (1996) showed that at 5°C the Col3a1 product remained largely in the random coil conformation and is thus essentially non-gelling. This is in accordance with the absence of helix-stabilizing hydroxyprolines, as confirmed by determination of the amino acid composition (data not shown).

Production of Col1a1-1 and Col1a1-2 gelatins

A 1·0 kb mouse *COL1A1* cDNA fragment encoding a 28 kDa gelatin (Col1a1-1) and a 1·8 kb *COL1A1* cDNA fragment encoding a 53 kDa gelatin (Col1a1-2), were cloned in pPIC9. The vectors pCOL1A1-1 and pCOL1A1-2 thus obtained were used to transform *P. pastoris*, after which representative transformants were selected for fermentation.

Optimized COL1A1-1 fermentations involved growth at pH 3·0 and supplementation of the medium with casamino acids. Expression levels ranged from 2–3 g/l in the clarified broth. SDS–PAGE results are shown in Figure 3A. Apart from minor degradation products, several distinct major bands can be observed. The highest of these major bands has an apparent molecular weight of 38 kDa, which corresponds well to the apparent molecular weight expected for full-length Col1a1-1. Although the results obtained from fermentations at pH 5·0 were not significantly different from those at pH 3·0 (data not shown), the presence of casamino acids in the medium appeared to be important. The full-length band was barely detectable in fermentations that did not contain casamino acids (Figure 3B).

A *P. pastoris* COL1A1-2 transformant was fermented under the same conditions used in the optimized COL1A1-1 fermentations. Expression levels ranged from 2–3 g/l in the clarified broth. SDS–PAGE showed a similar banding pattern to that obtained for COL1A1-1 (Figure 4). Interestingly, the lowest two major bands of the Col1a1-2 gelatin have the same apparent molecular weight as the lowest two bands of Col1a1-1. The highest Col1a1-2 band has the expected apparent molecular weight of 74 kDa.

Analysis of Col1a1 protein fragments and site-directed mutagenesis

In order to establish their identity, the Col1a1 protein fragments were subjected to N-terminal protein sequencing. Sequences corresponding to

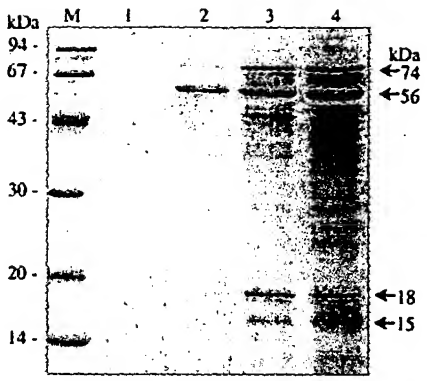

Figure 4. SDS–PAGE of a COL1A1-2 fermentation at pH 3·0 supplemented with casamino acids. Lanes 1–4: 3 µl culture supernatant, collected at 24, 50, 96 and 164 h, respectively. Lane M: molecular weight marker.

both the N-terminus (double underlines in Figure 5) and to internal positions were obtained (single underlines in Figure 5). The sequences corresponding to the N-termini of full-length Coll a1-1 and Coll a1-2 revealed a Glu–Ala extension. Such an extension of one or more Glu–Ala pairs, derived from the α-factor prepro secretory signal, is known to be present in some cases due to incomplete processing by the *STE13* gene product (Vedvick *et al.*, 1991). In the case of Coll a1, this may well be caused by the presence of a proline residue immediately C-terminal to the Glu–Ala–Glu–Ala spacer (Van den Bergh *et al.*, 1987).

Based on their N-terminal sequences and their molecular weights as determined by SDS–PAGE (corrected for the anomalous migration rate of collagenous proteins), the protein fragments could be assigned to distinct parts of the theoretical Coll a1 sequence (Table 1). Interestingly, the N-terminal sequences of the internal Coll a1 peptides were preceded in the theoretical sequence by the sequence Met–Gly–Pro–Arg (Figure 5, residues 38–41 and 122–125). This sequence occurs only at these two positions in Coll a1-1 and Coll a1-2, suggesting that it was possibly recognized by a specific endopeptidase. In order to verify this hypothesis, the cleavage sites were altered by site-directed mutagenesis. To preserve the native amino acid composition of Coll a1, the sequences were converted to Arg–Gly–Pro–Met, yielding plasmids pCOL1A1-1* and pCOL1A1-2*, respectively. *P. pastoris* strain GS115 was transformed with plasmids pCOL1A1-1* and pCOL1A1-2* and fermentations were performed as described for COL1A1-1 and COL1A1-2. SDS–PAGE of the culture supernatant shows that, aside from minor degradation products, one major band of the expected full-length size is formed for COL1A1-1* as well as COL1A1-2* (Figure 6). The N-terminal sequences of the full-length bands were determined and were found to be identical to the expected N-terminus of intact Coll a1, although again extended with Glu–Ala.

Alternative recombinant gelatin production strategies

In order to investigate whether the Mut phenotype had an effect on productivity or proteolysis, Mut^S COL3A1 and COL1A1-1 transformants of GS115 were generated. Fermentation of these transformants at pH 3·0 did not show any reduction in proteolysis and did not improve yield (data not shown).

It has been reported that the use of the protease A-deficient *P. pastoris* strain SMD1168 can sometimes reduce proteolysis (Sreekrishna and Kropp, 1996). To verify its applicability for the production of recombinant gelatin, Mut^+ COL3A1 and

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   1  PMGPSGPRGL PGPPGAPGPQ GFQGPPGEPG EPGGSGPMGP RGPPGPPGKN GDDGEAGKPG
  61  RPGERGPPGP QGARGLPGTA GLPGMKGHRG FSGLDGAKGD AGPAGPKGEP GSPGENGAPG
 121  QMGPRGLPGE RGRPGPPGTA GARGNDGAVG AAGPPGPTGP TGPPGFPGAV GAKGEAGPQG
 181  ARGSEGPQGV RGEPGPPGPA GAAGPAGNPG ADGQPGAKGA NGAPGIAGAP GFPGARGPSG
 241  PQGPSGPPGP KGNSGEPGAP GNKGDTGAKG EPGATGVQGP PGPAGEEGKR GARGEPGPSG
 301  LPGPPGERGG PGSRGFPGAD GVAGPKGPSG ERGAPGPAGP KGSPGEAGRP GEAGLPGAKG
 361  LTGSPGSPGP DGKTGPPGPA GQDGRPGPAG PPGARGQAGV MGFPGPKGTA GEPGKAGERG
 421  LPGPPGAVGP AGKDGEAGAQ GAPGPAGPAG ERGEQGPAGS PGFQGLPGPA GPPGEAGKPG
 481  EQGVPGDLGA PGPSGARGER GFPGERGVQG PPGPAGPRGN NGAPGNDGAK GDTGAPGAPG
 541  SQGAPGLQGM PGERGAAGLP GPKGDRGDAG PKGADGSPGK DGARGLTGPI GPPLE
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Figure 5. Theoretical Coll a1-2 protein sequence. Coll a1-1 shares residues 1–308 with Coll a1-2 and ends with Leu–Glu, as does Coll a1-2. The N-terminal sequences obtained for the Coll a1 protein fragments are underlined. Double-underlining at the N-terminus indicates that two fragments gave the same N-terminal sequence, both of which had a Glu–Ala extension.

Table 1. Apparent and theoretical molecular weights of Coll a1 fragments.

Recombinant gelatin	Apparent molecular weight (kDa)	Corrected molecular weight (kDa)	Assigned residues	Theoretical molecular weight (kDa)
Coll a1-1	38	27	1–310	28
	24	17	126–310	16
	18	13	1–125	12
	15	11	42–125	8
Coll a1-2	74	53	1–595	53
	56	40	126–595	42
	18	13	1–125	12
	15	11	42–125	8

Apparent molecular weights are values derived by comparison with globular molecular weight marker proteins. Corrected molecular weights are the apparent molecular weights divided by 1·4 to account for the aberrant migration rate of collagenous proteins, as compared to globular proteins. Theoretical molecular weights were calculated from the indicated parts of the sequence. Note that both Coll a1 types share corresponding protein fragments, having apparent molecular weights of 15 and 18 kDa.

COL1A1-1 transformants of this strain were constructed and fermented at pH 3·0. SDS–PAGE of the culture supernatants revealed no decreased proteolysis or improved productivity (data not shown).

To further increase expression levels in *P. pastoris* GS115, the *COL3A1* DNA fragment was cloned into the vector pPIC9K, which allows the selection of multicopy transformants (Scorer *et al.*, 1994). A series of transformants containing approximately 1–15 copies of the vector was obtained, as determined by quantitative dot-blot analysis (data not shown). A shaking flask production expression experiment showed that productivity appeared to increase with increasing gene copy number. To investigate this further, transformants containing 5 or 15 copies were fermented. The 5-copy transformant produced 11·3 g gelatin/litre and the 15-copy transformant produced 14·8 g gelatin/litre of clarified broth. Thus, productivity was considerably improved as compared to the single-copy COL3A1 transformant.

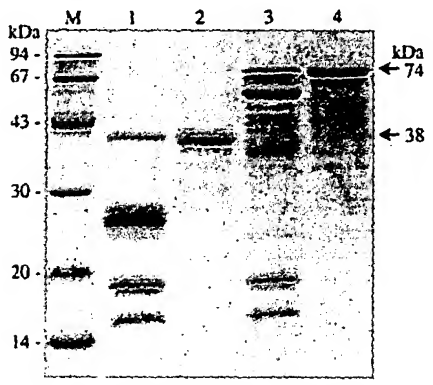

Figure 6. SDS–PAGE comparison of Coll a1-1, Coll a1-1*, Coll a1-2 and Coll a1-2*. All fermentations were performed at pH 3·0 and were supplemented with casamino acids. Culture supernatants were collected at the end of each fermentation and purified by acetone fractionation (see Materials and Methods). The apparent molecular weights of the full-length bands are indicated. Lanes 1–4: Coll a1-1, Coll a1-1*, Coll a1-2 and Coll a1-2*, respectively. Lane M: molecular weight marker.

DISCUSSION

This paper describes the extracellular production of recombinant gelatins in *P. pastoris*. Different strategies were used to reduce the proteolytic degradation of this extremely vulnerable unfolded protein.

Initial fermentations of COL3A1 at pH 5·0 resulted in severe degradation of the full-length protein. This effect could be minimized by performing the fermentation at pH 3·0, suggesting proteolysis by extracellular proteases. In contrast, degradation of Coll a1-1 and Coll a1-2 could not be repressed by fermenting a pH 3·0. These gelatins were degraded to several distinct degradation products from the onset of fermentation. In order

to obtain detectable amounts of the full-length Col1a1-1 band, it was necessary to supplement the fermentation medium with casamino acids. This is in contrast with COL3A1 fermentations, where the use of casamino acids did not have any effect on degradation. Despite the beneficial effect of adding casamino acids to the medium, which has been reported to repress extracellular proteases (Clare *et al.*, 1991a), the distinct major Col1a1 degradation products were still formed. Their formation at the onset of fermentation suggests that some type of proteolytic cleavage may take place intracellularly. This notion is supported by the finding that incubation of the (clarified as well as non-clarified) Col1a1-1 fermentation broth at 30°C for many days did not lead to degradation of the full-length band to the smaller species (data not shown).

N-terminal sequence analysis of the Col1a1 bands showed that the shorter species were internal fragments. The internal sequences were different from each other, but both were preceded in the theoretical Col1a1 sequence by Met–Gly–Pro–Arg. We hypothesized that Met–Gly–Pro–Arg might represent a motif that is specifically cleaved by an endopeptidase. Indeed, conversion in Col1a1-1 and Col1a1-2 of the Met–Gly–Pro–Arg sequences by site-directed mutagenesis to Arg–Gly–Pro–Met resulted in the disappearance of the major degradation products in favour of the full-length proteins. Basic residues or pairs of basic residues are a well-known recognition site for proteolytic processing of prohormones in eurkaryotes (for review, see Van de Ven *et al.*, 1993). The *S. cerevisiae* subtilisin-like Kex2 protease was the first known enzyme with a cleavage specificity for paired basic residues (Julius *et al.*, 1984). It has been suggested that Kex2 is also able to cleave the mono-arginylic sequence Pro–Arg (Mizuno *et al.*, 1989). If Kex2 were to recognized Pro–Arg in the Met–Gly–Pro–Arg sequence, one might expect cleavage of all occurrences of Pro–Arg, in view of the absence of folded structure in non-hydroxylated gelatin. However, Col1a1 and Col3a1 do contain the sequences Ala–Gly–Pro–Arg, Ser–Gly–Pro–Arg, Gln–Gly–Pro–Arg, Pro–Gly–Pro–Arg and a dibasic Glu–Gly–Lys–Arg, which are apparently not cleaved, or at least not to the extent of the Met–Gly–Pro–Arg sites. Furthermore, the conversion of residues 38–41 (Figure 5) to Arg–Gly–Pro–Met coincidentally generates the sequence Ser–Gly–Pro–Arg, which is evidently not substantially cleaved, since the mutations were effective in enabling the expression of full-length Col1a1-1 and Col1a1-2. Thus, we conclude that neighbouring residues are involved in recognition by the protease. As both the +1 and the −3 position (relative to the site of cleavage) are occupied by Gly in all of the above mentioned Pro–Arg sites in the recombinant gelatins, it is likely that the residue at the −4 position is the determining factor for cleavage. Brenner and Fuller (1992) mention that known Kex2-cleaved sites appear to contain aliphatic residues at the −4 position. Elaborating on this, we observe that the −4 position in known cleaved proteins is occupied by the more hydrophobic [Leu–Ile–Val–Met] subset of the aliphatic residues (Singh *et al.*, 1983; Zhu *et al.*, 1992; Ledgerwood *et al.*, 1995; Rourke *et al.*, 1997). Although probably constituting the preferred Kex2 substrates, a basic or proline residue at the −2 position may not be strictly necessary (Brenner and Fuller, 1992; Rourke *et al.*, 1997). We therefore speculate that the mono-arginylic sequence Met–Gly–Pro–Arg is cleaved by Kex2 or a Kex2-like protease, which may recognize sequences conforming to the motif [Leu–Ile–Val–Met]–Xaa–Yaa–Arg. Accordingly, this motif occurs only at the two Met–Gly–Pro–Arg sites in the Col1a1 sequence, in contrast with a mere Pro–Arg motif. It will require the construction of a *P. pastoris KEX2* gene disruptant to determine whether this protease is indeed involved in the cleavage at Met–Gly–Pro–Arg.

In an attempt to further enhance the yield and integrity of the recombinant gelatins, several strategies were employed. The use of Mut^S transformants or transformants of the protease A deficient strain SMD1168 did not lead to enhanced productivity or reduced degradation. Productivity was, however, substantially improved by the use of multicopy transformants. A transformant bearing approximately 15 copies of the pCOL3A1 vector produced up to 14·8 g gelatin/litre extracellular medium. To our knowledge, this represents an unprecedented high yield of secreted heterologous proteins in *P. pastoris*.

In view of the high productivity obtained, *P. pastoris* is apparently well able to cope with the extraordinary sequence of gelatin, which is highly repetitive and codes for some 33% Gly and 22% Pro. The codon usage for these amino acids in the rat *COL3A1* sequence fits that of *P. pastoris* highly expressed genes (Sreekrishna and Kropp, 1996) fairly well. The codon usage of the *COL1A1-1* and *COL1A1-2* sequences, however, differs considerably. In the *COL1A1-1* sequence, glycine is

encoded by the codon GGC in 28% of occurrences, while this is only 3% in the *P. pastoris* highly expressed genes. In the case of the proline encoding codon CCC this is 31% and 4%, respectively. Possibly, this codon usage may account for the lower expression levels of Col1a1-1 and Col1a1-2 as compared to Col3a1.

It has been suggested that the maximum level of protein secretion is ultimately determined by the protein-folding capacity of the endoplasmic reticulum (Parekh *et al.*, 1995; Parekh and Wittrup, 1997). Exceeding this capacity by the use of multicopy transformants is thought to result in the accumulation of unfolded proteins in the endoplasmic reticulum and a concomitant vast decrease in the level of expression due to physiological instability. In this respect, the unique characteristics of gelatin may provide an explanation for the exceptionally high secretion levels obtained with our multicopy transformants. As non-hydroxylated gelatin is unfolded, it will probably not drain the folding capacity of the endoplasmic reticulum. Also, in contrast with incorrectly folded globular proteins, it is not likely that the unfolded gelatin will aggregate and accumulate in the endoplasmic reticulum, due to its outstanding solubility. This notion is supported by the observation that lysed cells did not show significant intracellular retention of recombinant gelatin (data not shown).

It can be concluded that *P. pastoris* is a very suitable host for the production of recombinant gelatins. The levels of heterologous protein secretion were exceptionally high. Proteolytic degradation could be minimized despite the unfolded structure of gelatin. Given the ease of upscaling fermentations of *P. pastoris*, these findings open up possibilities for economical large-scale production of recombinant gelatins.

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